



#4

Our Ref.: 146.1169-CON-1

PROCESS FOR OXIDATION OF STEROIDS AND

GENETICALLY ENGINEERED CELLS USED THEREIN

ABSTRACT OF THE DISCLOSURE

An expression cassette, operable in a recombinant host, comprising a heterologous DNA coding sequence encoding a protein, which is functional, alone or in cooperation with one or more additional proteins, of catalyzing an oxidation step in the biological pathway for conversion of cholesterol into hydrocortisone, which step is selected from the group consisting of:

the conversion of cholesterol to pregnenolone;
the conversion of pregnenolone to progesterone;
the conversion of progesterone to 17 α -hydroxyprogesterone;
the conversion of 17 α -hydroxyprogesterone to cortexolone;
the conversion of cortexolone to hydrocortisone, and the corresponding control sequences effective in said host.

PRIOR APPLICATIONS

This application is a continuation-in-part of U.S. patent application Serial No. 054,185 filed April 26, 1993 which is a continuation of U.S. patent application Serial No. 474,857 filed October 30, 1990, now abandoned and of U.S. patent application Serial No. 002,608 filed January 11, 1993 which is a continuation of U.S. patent application Serial No. 474,798 filed July 16, 1990, now abandoned.

STATE OF THE ART

Δ^4 -pregnene-11 β ,17 α ,21-triol-3,20-dione (hydrocortisone) is an important pharmaceutical steroid, used for its pharmacological properties as a corticosteroid and as a starting compound for the preparation of numerous useful steroids, particularly other corticosteroids. Hydrocortisone is produced in the adrenal cortex of vertebrates and was originally obtained, in small amounts only, by a laborious extraction from adrenal cortex tissue. Only after structure elucidation were new production routes developed, characterized by a combination of chemical synthesis steps and microbiological conversions. Only because the starting compounds which are employed such as sterols, bile acids and sapogenins are abundant and cheap, the present processes afford a less expensive product, but these still are rather complicated. Several possibilities were envisaged to improve the present processes, and also biochemical approaches have been tried.

One attempt was to have a suitable starting steroid converted in an in vitro biochemical system using the isolated adrenal cortex proteins which are known to be responsible for the enzymatical conversion in vivo of steroids to hydrocortisone. However, the difficult isolation of the proteins and the high price of the necessary cofactors, appeared to be prohibitive for an economically attractive large scale process.

Another approach was to keep the catalyzing proteins in their natural environment and to have the adrenal cortex cells produce the desired hydrocortisone in a cell culture. But due to the low productivity of the cells, in practice, it appeared to be impossible to make such a biochemical process economically attractive.

The in vivo process in the adrenal cortex of mammals and other vertebrates constitutes a biochemical pathway, which starts with cholesterol and via various intermediate compounds eventually affords hydrocortisone (see figure 1). Eight proteins are directly involved in this pathway, five of them being enzymes, among which four cytochrome P₄₅₀ enzymes, and the other three being electron transferring proteins.

The first step is the conversion of cholesterol to 3 β -hydroxy-5-pregnene-20-one (pregnenolone). In this conversion, a mono-oxygenase reaction, three proteins are involved: side-chain

cleaving enzyme ($P_{450}SCC$, a heme-Fe-containing protein), adrenodoxin (ADX, a Fe_2S_2 containing protein) and adrenodoxin reductase (ADR, a FAD-containing protein).

Besides cholesterol as a substrate, the reaction further requires molecular oxygen and NADPH. Subsequently, pregnenolone is converted by dehydrogenation/isomerization to Δ^4 -pregnene-3,20-dione (progesterone). This reaction, catalyzed by the protein 3β -hydroxy steroid dehydrogenase/isomerase (3β -HSD), requires pregnenolone and NAD⁺.

To obtain hydrocortisone, progesterone subsequently is hydroxylated at three positions which conversions are catalyzed by mono-oxygenases. In the conversions of progesterone into 17α -hydroxy progesterone, two proteins are involved:

steroid 17α -hydroxylase ($P_{450}17\alpha$, a heme-Fe-containing protein) and NADPH cytochrome P_{450} reductase (RED, a FAD- and FMN-containing protein). The reaction consumes progesterone, molecular oxygen and NADPH.

For the conversion of 17α -hydroxyprogesterone into $17\alpha,21$ -dihydroxy- Δ^4 -pregnene-3,20-dione (cortexolone), also two proteins are needed: steroid-21-hydroxylase ($P_{450}C21$, a heme-Fe-containing protein) and the before-mentioned protein RED. The reaction consumes 17α -hydroxy progesterone, molecular oxygen and NADPH.

10084814.042602

In the conversion of cortexolone into hydrocortisone, three proteins are involved: steroid 11 β -hydroxylase (P₄₅₀11 β , a heme-Fe-containing protein), and the above mentioned proteins ADX and ADR.

As described above, cytochrome P₄₅₀ proteins are enzymes which are essential for the biochemical conversion of cholesterol to hydrocortisone. These enzymes belong to a larger group of cytochrome P₄₅₀ proteins (or shortly P₄₅₀ proteins). They have been encountered in prokaryotes (various bacteria) and eukaryotes (yeasts, molds, plants and animals). In mammals, high levels of P₄₅₀ proteins are found in the adrenal cortex, ovary, testes and liver.

Many of these proteins have been purified and are well characterized now. Their specific activity has been determined. Recently, a number of reviews on this subject have been published such as K. Ruckpaul and H. Rein (eds), "Cytochrome P₄₅₀" and P.R. Ortiz de Montellano (ed.) "Cytochrome P₄₅₀, structure, mechanism and biochemistry". Cytochrome P₄₅₀ proteins are characterized by their specific absorbance maximum at 450 nm after reduction with carbon monoxide. In prokaryotic organisms, the P₄₅₀ proteins are either membrane bound or cytoplasmatic. As far as the bacterial P₄₅₀ proteins have been studied in detail (e.g. P₄₅₀meg and P₄₅₀cam), it has been shown that a ferredoxin and a ferredoxin reductase are involved in the hydroxylating activity. For eukaryotic organisms, two types of P₄₅₀ proteins, I and II have been described. Their two

differences reside in:

1. subcellular localization, type I is localized in the microsomal fraction and type II is localized in the inner membrane of mitochondria;
2. the way the electrons are transferred to the P_{450} protein. Type I is reduced by NADPH via a P_{450} reductase, whereas Type II is reduced by NADPH via a ferredoxin-reductase (e.g. adrenodoxin reductase) and a ferredoxin (e.g. adrenodoxin).

According to EP-A-0,281,245, cytochrome P_{450} enzymes can be prepared from Streptomyces species and used for the hydroxylation of chemical compounds. The enzymes are used in isolated form, which is a rather tedious and expensive procedure.

JP-A-62,236,485 (Derwent 87-331,234) teaches that it is possible to introduce into Saccharomyces cerevisiae the genes of liver cytochrome P_{450} enzymes and to express them affording enzymes which may be used for their oxidation activity. However, in the above references, there is no indication of the use of cytochrome P_{450} enzymes for the preparation of steroid compounds.

OBJECTS OF THE INVENTION

It is an object of the invention to provide an improved biochemical pathway for the production of hydrocortisone and expression cassettes useful therein.

It is another object of the invention to provide recombinant host cells and their progeny containing said expression cassettes.

These and other objects and advantages of the invention will become obvious from the following detailed description.

THE INVENTION

The process of the invention for the preparation of hydrocortisone from sterols comprises culturing a recombinant cell in a nutrient medium, the recombinant host containing an expression cassette, operable in a recombinant host, comprising a heterologous DNA coding sequence encoding a protein, which is functional, alone or in cooperation with one or more additional proteins, of catalyzing an oxidation step in the biological pathway for conversion of cholesterol into hydrocortisone, which step is selected from the group consisting of:

- the conversion of cholesterol to pregnenolone;
- the conversion of pregnenolone to progesterone;
- the conversion of progesterone to 17 α -hydroxy-progesterone;

the conversion of 17 α -hydroxyprogesterone to cortexolone;
the conversion of cortexolone to hydrocortisone, and the
corresponding control sequences effective in said host.

The invention provides a multiplicity of expression cassettes for production of proteins necessary in the construction of a multigenic system for the one-step conversion of inexpensive steroid starting materials to more rare and expensive end products, wherein such conversion is carried out in native systems through a multiplicity of enzyme-catalyzed and cofactor-mediated conversions, such as the production of hydrocortisone from cholesterol. The expression cassettes of the invention are useful in the ultimate production of multigenic systems for conducting these multi-step conversions.

Accordingly, in one aspect, the invention is directed to an expression cassette effective in a recombinant host cell in expressing a heterologous coding DNA sequence, wherein said coding sequence encodes an enzyme which is able, alone or in cooperation with additional proteins, to catalyze an oxidation step in the biological pathway for the conversion of cholesterol to hydrocortisone. The expression cassettes of the invention, therefore, include those sequences capable of producing, in a recombinant host, the following proteins: side-chain cleaving enzyme (P₄₅₀SCC); adrenodoxin (ADX); adrenodoxin reductase (ADR); 3 β -hydroxy steroid dehydrogenase/isomerase (3 β -HSD); steroid 17 α -

hydroxylase ($P_{450}17\alpha$); NADPH cytochrome P_{450} reductase (RED); steroid-21-hydroxylase ($P_{450}C21$); and steroid 11β -hydroxylase ($P_{450}11\beta$).

In other aspects, the invention is directed to recombinant host cells transformed with these vectors or with the expression cassettes of the invention, to methods to produce the above enzymes and to use these enzymes for oxidation, to processes to use said host cells for specific oxidations in a culture broth and to pharmaceutical compositions containing compounds prepared by said processes.

BRIEF DESCRIPTION OF THE FIGURES

Abbreviations used in all figures: R_1 , EcoRI; H, HindIII; Sc, ScaI; P, PstI; K, KpnI; St, StuI; Sp, SphI; X, XbaI; N, NdeI; S, SmaI; Ss, SstI; R_v , EcoRV; S_1 , SacI; B, BamHI; S_{11} , SacII; Sal, SalI; Xh, XhoI; Pv, PvuII; Bg, BglII and M, MluI.

Figure 1 shows a schematic overview of the proteins involved in the succeeding steps in the conversion of cholesterol in hydrocortisone as occurring in the adrenal cortex of mammals.

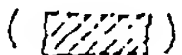
Figure 2 shows the construction of plasmid pGBSCC-1. The P_{450} SCC-sequences are indicated in a box ().

Figure 3 shows the insertion of a synthetically derived PstI/HindIII fragment containing the 5'-P₄₅₀SCC-sequences into the plasmid pTZ18R to obtain the plasmid pTZ synlead.

Figure 4 shows the construction of a full-length P₄₅₀SCC cDNA of synthetically (□) and by cDNA cloning (▨) derived P₄₅₀ SCC-sequences into pTZ18R to obtain pGBSCC-2.

Figure 5 shows the complete nucleotide sequence of plasmid-pBHA-1.

Figure 6 is a schematic representation of the construction of pGBSCC-3. P₄₅₀SCC DNA sequences from plasmid pGBSCC-2 were introduced into the Bacillus/E.coli shuttle plasmid pBHA-1. Filled in boxes are as indicated in the legend of Figure 4.

Figure 7 shows the introduction of a NdeI restriction site in combination with an ATG start codon before the P₄₅₀SCC-maturation site in pGBSCC-3 to obtain pGBSCC-4.

Figure 8 shows a physical map of pGBSCC-5 which is obtained by removal of E.coli sequences from the plasmid pGBSCC-4.

Figure 9 shows a Western-blot probed with anti-bodies against P₄₅₀SCC, demonstrating the P₄₅₀SCC expression of plasmid pGBSCC-5 introduced in B.subtilis (lane c) and B.licheniformis (lane f).

Control extracts from B.subtilis (lane c) and B.licheniformis are shown in lanes (a) and (d) resp.. For comparison also purified adrenal cortex P₄₅₀SCC (30 ng) was added to these control extracts (lanes (b) and (e) resp.).

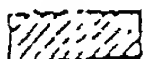
Figure 10 is a schematic representation of the construction of pGBSCC-17. The coding P₄₅₀SCC-DNA sequences from plasmid pGBSCC-4 were introduced into the E.coli expression vector pTZ18RN. The P₄₅₀ SCC-sequences are indicated in a box ().

Figure 11 shows the P₄₅₀ SCC expression of pGBSCC-17 in E.coli JM101.

- (a) SDS/PAGE and Coomassie brilliant blue staining of the cellular protein fractions (20 μ l) prepared from the E.coli control strain (lane 3) and E.coli transformants SCC-301 and 302 (lanes 1 and 2, resp.). 400 ng purified bovine P₄₅₀SCC (lane 4) is shown for comparison.
- (b) Western-blot analysis, probed with antibodies against P₄₅₀SCC of cellular protein fractions (5 μ l) prepared from the control strain E.coli JM101 (lane 2) and from the E.coli JM101 (lane 3) and SCC-302 (lane 4). 100 ng purified bovine P₄₅₀SCC (lane 1) is shown for comparison.

Figure 12 shows the construction of plasmid pUCG418.

Figure 13 shows the construction of the yeast expression

vector PGB950 by insertion of the promoter and terminator with multiple cloning sites (~~1722~~) of lactase in pUCG418. To derive pGBSCC-6, a synthetic SalI/XhoI fragment containing an ATG start codon and the codons for the first 8 amino acids of P₄₅₀SCC is inserted in pGB950.

Figure 14 is a schematic presentation showing the construction of the yeast P₄₅₀SCC-expression cassette pGBSCC-7.

Figure 15 shows a Western-blot probed with antibodies specific for the protein P₄₅₀SCC.

Blot A contains extracts derived from Saccharomyces cerevisiae 273-10B transformed with pGBSCC-10 (lane 1); from S.cerevisiae 273-10B as a control (lane 2); from Kluyveromyces lactis CBS 2360 transformed with pGBSCC-7 (lane 3) and from K.lactis CBS 2360 as a control (lane 4).

Blot B contains extracts derived from K.lactis CBS 2360 as a control (lane 1) and K.lactis CBS 2360 transformed with pGBSCC-15 (lane 2), with pGBSCC-12 (lane 3) or with pGBSCC-7 (lane 4).

Blot C contains extracts derived from S.cerevisiae 273-10B as a control (lane 1) transformed with pGBSCC-16 (lane 2) or with pGBSCC-13 (lane 3).

Figure 16 is a schematic presentation of the construction of the yeast expression vector pGBSCC-9 containing the isocytochrome CI (cyc-1) promoter from S.cerevisiae.

Figure 17 shows a construction diagram of the P_{450} SCC cDNA containing expression vector pGBSCC-10 for S.cerevisiae.


Figure 18 shows the construction of the P_{450} SCC expression vector pGBSCC-12 in which a synthetically derived DNA-fragment encoding the pre- P_{450} SCC sequence () is inserted 5' for the coding sequence of mature P_{450} SCC.

Figure 19 shows the construction of the pGBSCC-13. This P_{450} SCC expression cassette for S.cerevisiae contains the pre- P_{450} SCC cDNA sequence positioned 3' of the cyc-1 promoter of S.cerevisiae.


Figure 20 shows a schematic representation of the construction of the plasmids pGBSCC-14 and pGBSCC-15. The latter contains the P_{450} SCC coding sequence in frame with the cytochrome oxidase VI pre-sequence () .


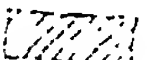
Figure 21 shows the construction of the plasmid pGBSCC-16. In this plasmid, the cytochrome oxidase VI presequence () of S.cerevisiae fused to the coding P_{450} SCC sequence is positioned 3' of the cyc-1 promoter.

Figure 22 shows the physical maps of the plasmids pGB17 α -1 (A) and pGB17 α -2 (B) containing the 3' 1.4 kb fragment and the 5' 345 bp fragment () of P_{450} 17 α cDNA, resp.. In pGB17 α -3 (C) containing the full length P_{450} 17 α cDNA sequence, the position of

the ATG start codo. is indicated.

Figure 23 shows the mutation of pGB17 α -3 by in vitro mutagenesis. The obtained plasmid pGB17 α -4 contains a SalI restriction site followed by optimal yeast translation signals just upstream the ATG initiation codon.

Figure 24 is a schematic view of the construction of the yeast P₄₅₀17 α expression cassette pGB17 α -5.

Figure 25 shows the mutation of pGB17 α -3 by in vitro mutagenesis. The obtained plasmid pGB17 α -6 contains an NdeI restriction site at the ATG-initiation codon.

Figure 26 is a schematic representation of the construction of pGB17 α -7. P₄₅₀17 α cDNA sequences from plasmid pGB17 α -6 were introduced into the Bacillus/E.coli shuttle plasmid pBHA-1.

Figure 27 shows a physical map of pGB17 α -8 which is obtained by removal of E.coli sequences from the plasmid pGB17 α -7.

Figure 28 shows physical maps of pGBC21-1 and 2, containing an 1.53 kb 3'-P₄₅₀C21 cDNA and a 540 bp 5'-P₄₅₀ cDNA EcoRI fragment, respectively, in the EcoRI-site of the cloning vector pTZ18R.

Figure 29 shows the in vitro mutagenesis by the polymerase

chain reaction (PCR) of pGBC21-2 to introduce EcoRV and NdeI restriction sites upstream the P₄₅₀C21 ATG-initiation codon, followed by molecular cloning into the cloning vector pSP73 to derive pGBC21-3.

Figure 30 is a schematic view of the construction of pGBC21-4, containing the full-length P₄₅₀C21 cDNA sequence.

Figure 31 is a schematic representation of the construction of pGBC21-5. The P₄₅₀C21 cDNA sequence from plasmid pGBC21-4 was introduced into the Bacillus/E.coli shuttle plasmid pBHA-1.

Figure 32 shows a physical map of pGBC21-6 which is obtained by removal of E.coli sequences from the plasmid pGBC21-5.

Figure 33 shows the mutation of pGBC21-2 by in vitro mutagenesis. The obtained plasmid pGBC21-7 contains a SalI restriction site followed by optimal yeast translation signals just upstream the ATG initiation codon.

Figure 34 represents the construction of pGBC21-8, containing a full-length P₄₅₀C21 cDNA with modified flanking restriction sites suitable for cloning into the yeast expression vector.

Figure 35 is a schematic presentation showing the construction of the yeast P₄₅₀C21-expression cassette pGBC21-9.

Figure 36 shows the in vitro mutagenesis by the polymerase chain reaction of pGB11 β -1 to introduce appropriate flanking restriction sites and an ATG initiation codon to the full-length P₄₅₀11 β cDNA sequence, followed by molecular cloning into the Bacillus/E.coli shuttle vector pBHA-1 to derive the plasmid pGB11 β -2.

Figure 37 shows the in vitro mutagenesis by the polymerase chain reaction of pGB11 β -1 to introduce appropriate flanking restriction sites and an ATG initiation codon to the full-length P₄₅₀11 β cDNA sequence, followed by molecular cloning into the yeast expression vector pGB950 to derive the plasmid pGB11 β -4.

Figure 38 is a schematic view of the molecular cloning of the ADX cDNA sequence from a bovine adrenal cortex polyA⁺RNA/cDNA mixture by the polymerase chain reaction method. The cDNA sequence encoding the mature ADX protein was inserted into the appropriate sites of the yeast expression vector pGB950 to obtain the plasmid pGBADX-1.

Figure 39 shows a Western-blot probed with antibodies against ADX, demonstrating the ADX expression of plasmid pGBADX-1 in K.lactis CBS 2360 transformants ADX-101 and 102 (lanes 4 and 5, resp.). Extract of control strain K.lactis CBS 2360 is shown in lane 3. For comparison, also purified adrenal cortex ADX (100 ng) is supplied to the gel in lane 1.

Figure 40 shows the in vitro mutagenesis by the polymerase chain reaction of pGBADR-1 to introduce appropriate flanking restriction sites and an ATG-initiation codon to the full-length ADR cDNA sequence, followed by molecular cloning into the yeast expression vector pGB950 to derive pGBADR-2.

Figure 41 shows a physical map of the expression cassette pGB17 α -5.

Figure 42 shows a physical map of the expression cassette pGBC21-9.

Figure 43 represents the construction of the expression cassette pGB17 α /C21-1, containing the coding sequence for P₄₅₀^{17 α} and P₄₅₀^{C21}, both driven by the lactase promoter.

Figure 44 shows a physical map of the plasmid pTG7457.

Figure 45 shows a physical map of the plasmid pTG7453.

Figure 46 shows a physical map of the plasmid pTG10014.

Figure 47 shows a physical map of the plasmid pTG10004.

Figures 48 and 49, respectively show a physical map of the plasmid pTG10031 and pTG10033.

Figure 50 shows a physical map of the plasmid pTG10013.

Figures 51 and 52, respectively show a physical map of the plasmid pTG10041 and pTG10042.

Figures 53 and 54, respectively show a physical map of the plasmid pTG10045 and pTG10046.

Figures 55 and 56, respectively show a physical map of the plasmid pTG10064 and pTG10065.

Figure 57 is RP-HPLC analysis of Example 34.

The invention comprises the preparation and culturing of cells which are suited to be used in large scale biochemical production reactors and the use of these cells for the oxidation of compounds and particularly for the production of steroids, shown in Figure 1. Each of the depicted reactions can be carried out separately. Also interchange of steps in a multi-step reaction is included in the invention. Micro-organisms are preferred hosts but other cells may be used as well as cells of plants or animals, optionally applied in a cell culture or in the tissue of living transgenic plants or animals.

The cells of the invention are obtained by the genetic

transformation of suitable receptor cells, preferably cells of suited micro-organisms, with vectors containing DNA sequences encoding the proteins involved in the conversion of cholesterol to hydrocortisone, comprising side-chain cleaving enzyme ($P_{450}SCC$), adrenodoxin (ADX), adrenodoxin reductase (ADR), 3β -hydroxy-steroid dehydrogenase/isomerase (3β -HSD), steroid- 17α -hydroxylase ($P_{450}17\alpha$), NADPH cytochrome P_{450} reductase (RED), steroid-21-hydroxylase ($P_{450}C21$) and steroid- 11β -hydroxylase ($P_{450}11\beta$). Some host cells may already produce on their own one or more of the necessary proteins at a sufficient level and therefore have to be transformed with the supplementary DNA sequences only. Such possible own proteins are ferredoxin, ferredoxin reductase, P_{450} -reductase, and 3β -hydroxy-steroid dehydrogenase/isomerase.

For retrieval of the sequences which encode proteins which are involved in the conversion of cholesterol to hydrocortisone, suitable DNA sources have been selected. An appropriate source for the retrieval of DNA encoding all proteins involved in the conversion of cholesterol to hydrocortisone is the adrenal cortex tissue of vertebrates e.g. bovine adrenal cortex tissue. Also from various micro-organisms, the relevant DNA can be retrieved, e.g. from Pseudomonas testosteroni, Streptomyces griseocarneus or Brevibacterium sterolicum for DNA encoding the 3β -hydroxy-steroid dehydrogenase/isomerase and from Curvularia lunata or Cunninghamella blakesleeana for DNA encoding proteins involved in the 11β -hydroxylation of cortexolone. The DNA-sequences coding for

the proteins bovine P₄₅₀SCC, bovine P₄₅₀11 β or a microbial equivalent protein, bovine adrenodoxin, bovine adrenodoxin reductase, 3 β -hydroxy-steroid dehydrogenase/isomerase of bovine or microbial origin, bovine P₄₅₀17 α , bovine P₄₅₀C21 and NADPH cytochrome P₄₅₀ reductase of bovine or microbial origin, were isolated according to the following steps:

1. Eukaryotic sequences (cDNA's)

- a. Total RNA was prepared from appropriate tissue.
- b. PolyA⁺ containing RNA was transcribed into double stranded cDNA and ligated into bacteriophage vectors.
- c. The obtained cDNA library was screened with ³²P-labeled oligomers specific for the desired cDNA or by screening an isopropyl- β -D-thiogalactopyranoside (IPTG)-induced lambda-gt11 cDNA library using a specific (¹²⁵I-labeled) antibody.
- d. cDNA inserts of positive plaque forming units (pfu's) were inserted into appropriate vectors to verify:
 - the entire length of the cDNA by nucleotide sequencing.

2. Prokaryotic genes

- a. Genomic DNA was prepared from an appropriate micro-organism.
- b. To obtain a DNA library, DNA fragments were cloned into appropriate vectors and transformed to an appropriate

E.coli host.

- c. The DNA library was screened with ^{32}P -labeled oligomers specific for the gene of interest or by screening an IPTG-induced lambda-gt11 cDNA library using a specific (^{125}I -labeled) antibody.
- d. Plasmids of positive colonies were isolated and inserted DNA fragments subcloned into appropriate vectors to verify:
 - the entire length of the gene.

Note: According to an improved method, the particular cDNA (eukaryotic sequences) or gene (prokaryotic sequences) was amplified using two specific oligomers by the method known as the polymerase chain reaction (PCR) (Saiki et al, Science, Vol. 239, pp. 487-491, 1988). Subsequently, the amplified cDNA or DNA was inserted into the appropriate vectors.

According to one aspect of the invention, suitable expression cassettes are provided in which the heterologous DNA isolated by the previous procedure is placed between suitable control sequences for transcription and translation, which enables the DNA to be expressed in the cellular environment of a suitable host, affording the desired protein or proteins. Optionally, the initiation control sequences are followed by a secretion signal sequence.

Suitable control sequences have to be introduced together with

the structural DNA by said expression cassettes. Expression is made possible by transformation of a suitable host cell with a vector containing control sequences which are compatible with the relevant host and are in operable linkage to the coding sequences of which expression is desired.

Alternatively, suitable control sequences present in the host genome are employed. Expression is made possible by transformation of a suitable host cell with a vector containing coding sequences of the desired protein flanked by host sequences enabling homologous recombination with the host genome in such a manner that host control sequences properly control the expression of the introduced DNA.

As is generally understood, the term control sequences comprises all DNA segments which are necessary for the proper regulation of the expression of the coding sequence to which they are operably linked, such as operators, enhancers and, particularly, promoters and sequences which control the translation.

The promoter may or may not be controllable by regulating its environment. Suitable promoters for prokaryotes include, for example, the trp promoter (inducible by tryptophan deprivation), the lac promoter (inducible with the galactose analog IPTG), the β -lactamase promoter, and the phage derived P_L promoter (inducible by

temperature variation). Additionally, especially for expression in Bacillus, useful promoters include those for alpha-amylase, protease, Spo2, spac and ø105 and synthetic promoter sequences. A preferred promoter is the one depicted in Figure 5 and denoted with "HpaII".

Suitable promoters for expression in yeast include the 3-phospho-glycerate kinase promoter and those for other glycolytic enzymes, as well as promoters for alcohol dehydrogenase and yeast-phosphatase. Also suited are the promoters for transcription elongation factor (TEF) and lactase. Mammalian expression systems generally employ promoters and the SV40 promoter but they also include regulatable promoters such as the metallothionein promoter, which is controlled by heavy metals or gluco-corticoid concentration. Presently, viral-based insect cell expression systems are also suited, as well as expression systems based on plant cell promoters such as the nopaline synthetase promoters.

Translation control sequences include a ribosome binding site (RBS) in prokaryotic systems, whereas in eukaryotic systems translation may be controlled by a nucleotide sequence containing an initiation codon such as AUG.

In addition to the necessary promoter and the translation control sequence, a variety of other control sequences, including those regulating termination (for example, resulting in

polyadenylation sequences in eukaryotic systems) may be used in controlling expression. Some systems contain enhancer elements which are desirable, but mostly not obligatory, in effecting expression.

The invention also discloses expression cassettes containing still another heterologous coding sequence encoding an enzyme which catalyzes, alone or in cooperation with one or more additional proteins, another step of the pathway of Figure 1.

A group of vectors denoted with pGBSCC-n, where "n" is any integer from 1 to 17, is especially developed for the DNA encoding the P_{450}^{SCC} enzyme.

Another group of vectors denoted with pGB17 α -n, where "n" is any integer from 1 to 5, is especially developed for the DNA encoding the $P_{450}^{17\alpha}$ enzyme.

A further group of vectors denoted with pGBC21-n, where "n" is any integer from 1 to 9, is especially developed for the DNA encoding the P_{450}^{C21} enzyme.

Still another group of vectors denoted with pGB11 β -n, where "n" is any integer from 1 to 4, is especially developed for the DNA encoding the $P_{450}^{11\beta}$ enzyme.

According to a further aspect of the invention, suitable host cells have been selected which accept the vectors of the invention and allow the introduced DNA to be expressed. When culturing the transformed host cells, the proteins involved in the conversion of cholesterol to hydro-cortisone appear in the cell contents. The presence of the desired DNA can be proven by DNA hybridizing procedures, their transcription by RNA hybridization, their expression by immunological assays and their activity by assessing the presence of oxidized products after incubation with the starting compound in vitro or in vivo.

Transformed microorganisms are preferred hosts, particularly bacteria (more preferably Escherichia coli and Bacillus and Streptomyces species) and yeasts (such as Saccharomyces and Kluyveromyces). Other suitable host organisms are found among plants and animals, comprising insects, of which the isolated cells are used in a cell culture, such as COS cells, C₁₂₇ cells, CHO cells, and Spodoptera frugiperda (Sf9) cells. Alternatively, a transgenic plant or animal is used.

A particular type of recombinant host cells are the ones in which either two or more expression cassettes of the invention have been introduced or which have been transformed by an expression cassette coding for at least two heterologous proteins, enabling the cell to produce at least two proteins involved in the pathway of Figure 1.

1000434-043602

A major feature of the invention is that the prepared novel cells are not only able to produce the proteins involved in the oxidative conversion of steroids resulting eventually into hydrocortisone, but also to use these proteins on the spot in the desired oxidative conversion of the corresponding substrate compound added to the culture liquid. Steroids are preferred substrates. The cells transformed with the heterologous DNA are especially suited to be cultured with the steroids mentioned in Figure 1, including other sterols such as β -sitosterol. As a result, oxidized steroids are obtained.

Depending on the presence in the host cell of a multiplicity of heterologous DNA encoding proteins involved in the pathway of Figure 1, several biochemical conversions result comprising the side-chain cleaving of a sterol and/or oxidative modifications of C11, C17, C3 and C21. Therefore, the expression cassettes of the invention are useful in constructing a multigenic system which can effect successive intra-cellular transformations of the multiple steps in the sequence as depicted in Figure 1. It may be necessary to introduce into the desired host expression cassettes which encode in their entirety the required proteins. In some instances, one or more of the proteins involved in the pathway may already be present in the host as a natural protein exerting the same activity. For example, ferredoxin, ferredoxin reductase and P_{450} reductase may already be present in the host. Under those circumstances, only the remaining enzymes must be provided by

recombinant transformation.

As an alternative to biochemical conversions in vivo, the proteins involved in the conversion of cholesterol to hydrocortisone are collected, purified as far as necessary, and used for the in vitro conversion of steroids in a cell free system, e.g. immobilized on a column. Alternatively, the more or less purified mixture containing one or more enzymes of the pathway is used as such for steroid conversion. One exemplified host contains DNA encoding two heterologous proteins viz. the enzyme $P_{450}SCC$ and the protein ADX necessary for the production of pregnenolone. In comparison with a host with only $P_{450}SCC$ DNA, the yield of pregnenolone in a cell-free extract after adding ADR, NADPH and cholesterol is considerably improved.

The present invention provides expression cassettes necessary for the construction of a one-step production process for several useful steroids. Starting from cheap and abundantly available starting compounds, it is especially suited for the production of hydrocortisone and intermediate compounds. The invention renders obsolete traditional expensive chemical reactions. Intermediate compounds need not be isolated. Apart from the novel host cells, the processes used for culturing these cells on behalf of steroid conversions are analogous to bio-technological procedures well known in the art.

10084944-042602

It has now been accomplished to clone in one host organism, the genes which code for the proteins which are able to catalyze two separate oxidations on the steroid molecule and particularly for the proteins shown in Figure 1. In particular, it has been realized to clone the proteins responsible for the steroid 17 α -hydroxylation and for the steroid C21-hydroxylation in one and the same host organism and to have said host organism express said proteins in a functional form. Moreover, in another aspect of the invention, a process is provided in which said transformed micro-organisms when grown in a fermentation medium oxidize a steroid substrate present in the medium simultaneously at two different positions of the steroid molecule. In particular, a one-step process is achieved for the introduction of the 17 α - as well as the 21-hydroxyl group.

A preferred host organism is Kluyveromyces lactis or is Saccharomyces cerevisiae, but other host organisms and in particular micro-organisms, especially those previously mentioned, can be used. More particularly, the micro-organisms are suitable which have been described above for cloning and expressing the genes of the biochemical pathway as shown in Figure 1.

One way to prepare a host able to carry out a multiple steroid oxidation is to transform the host with two or more vectors, each containing the gene for one oxidation step. One exemplified transformed host contains DNA encoding the P₄₅₀ 17 α and 3 β -HSDH proteins. Another way is to have the host transformed by one vector containing an expression

1006464-042602

cassette with all genes coding for the proteins necessary for the desired multiple oxidation reaction. According to the invention, the expression cassette contains at least two structural genes, each flanked by proper control sequences. One exemplified expression cassette contains DNA encoding the proteins P₄₅₀17 α and P₄₅₀-C21 (pGB17- α /C21-1).

Using the method of the invention it is possible, using methods known in the art, to prepare analogous expression cassettes and host cells containing them, with which it is possible to carry out other multiple steroid oxidations and eventually the conversion of cholesterol into hydrocortisone in a single fermentation process.

In another embodiment of the invention, the 3 β -hydroxy-5-ene steroid dehydrogenase and 5-ene-4-ene steroid isomerase (3 β HSDH) form a bifunctional enzyme which catalyzes two independant reactions transforming pregnenolone into progesterone. The protein of 42kD is encoded by a single open reading frame. cDNA's and/or genes have been cloned from human (Lachance et al., J. Biol. Chem. 265 (1990) p 20469-20475 ; Lachance et al., DNA Cell Biol. 10 (1991) p 701-711), bovine (Zhao et al., FEBS lett. 259 (1989) p 153-157) and rat (Zhao et al., J. Biol. Chem. 266 (1990) p 583-593) ; Simard J. et al., J. Biol. Chem. 266 (1991) p 14842-14845). The determined N-terminus corresponds to the deduced amino acid sequence. In human, two types have been described: type I 3 β HSDH

has been isolated from placenta and is also expressed in skin; type II 3β HSDH was isolated from adrenals and gonads. Nucleotide homologies between exons 1 till 4 are 77.1, 91.8, 94 and 94% respectively and 94% at the amino acid level. After expression in HeLa cells of the respective cDNA's, it was found that the type I enzyme is more active (V_{max}/K_m) on pregnenolone, DHEA (dehydroepiandrosterone) and DHT (dihydrotestosterone). This is primarily due to a lower K_m , e.g. for pregnenolone (0.24 versus 1.2 μM). Even in the absence of a mitochondrial targetting sequence, the 3β HSDH enzymes are known as membrane-associated proteins, located in microsomal as well as in mitochondrial membranes.

In the following examples, there are described several preferred embodiments to illustrate the invention. However, it should be understood that the invention is not intended to be limited to the specific embodiments.

Example 1

Molecular cloning of a full-length cDNA encoding the bovine cytochrome P₄₅₀ side chain cleavage enzyme (P₄₅₀SCC)

General cloning techniques as well as DNA and RNA analyses have been used as described in the handbook of T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, 1982. Unless described elsewhere, all DNA modifying enzymes, molecular cloning vehicles and E.coli strains were obtained from commercial suppliers and used according to the manufacturer's instructions. Materials and apparatus for DNA and RNA separation and purification were used according to instructions of the suppliers.

Bovine adrenal cortex tissue was prepared from freshly obtained bovine kidneys, quickly frozen in liquid nitrogen and stored at -80°C. From frozen bovine adrenal cortex, total cellular RNA was prepared as described by Auffrey et al (Eur. J. Biochem., Vol. 107, p. 303-314, 1980). Adrenal poly A+ RNA was obtained by heating the total RNA sample at 65°C before polyA selection on oligo(dT) chromatography.

209340-1990T
105494-042503

DNA's complementary to polyA⁺ RNA from bovine adrenal cortex were synthesized as follows: 10 µg of polyA⁺ RNA, treated with methylmercuric hydroxide were neutralized with β-mercaptoethanol and the mixture was adjusted to 50 mM Tris/HCl (pH 8.3 at 42°C), 40 mM KCl, 6 mM MgCl₂, 10 mM DTT, 3000 U RNasin/ml, 4 mM Na₄P₂O₇, 50 µg actinomycine D/ml, 0.1 mg oligo(dT₁₂₋₁₈)/ml, 0.5 mM dGTP, 0.5 mM dATP, 0.5 mM dTTP, 0.25 mM dCTP and 400 µCi α ³²P-dCTP/ml, all in a final volume of 100 µl. The mixture was put on ice for 10 minutes, heated for 2 minutes at 42°C and the synthesis was started by addition of 150 U AMV reverse transcriptase (Anglian Biotechnology Ltd.); incubation was performed for 1 hour at 42°C.

Second strand synthesis was performed by adding DNA polymerase and RNase H according to Gubler et al (Gene, Vol. 25, pp. 263-269, 1983). After treatment of the ds DNA with T4 DNA polymerase (BRL) to obtain blunt ends, decameric EcoRI linkers (Biolabs Inc.) were ligated to the ds DNA fragments. After digestion with EcoRI-linkers by Biogel A15 m (Bio-Rad) chromatography. Approximately 200 ng EcoRI-linker containing double stranded cDNA was ligated with 10 µg of EcoRI digested and calf intestine-phosphatase (Boehringer) treated with lambdagt11 vector DNA (Promega) by T4-DNA ligase (Boehringer) as described by Huynh et al. (In: "DNA cloning techniques: A practical approach", pp. 49-78, Oxford IRL-press, 1985). Phages obtained after in vitro packaging of the ligation mixture were used to infect the E.coli Y1090 host (Promega).

10084P4.042502

From this cDNA library, approximately 10^6 plaque forming units (pfu's) were screened with a ^{32}P -end labeled synthetic oligomer SCC-1 (5'-GGC TGA CGA AGT CCT GAG ACA CTG GAT TCA GCA CTGG-3'), specific for bovine P_{450}SCC DNA sequences as described by Morohashi et al. (Proc. Natl. Acad. Sci. USA, Vol. 81, pp. 4647-4651, 1984). Six hybridizing pfu's were obtained and further purified by two additional rounds of infection, plating and hybridization. The P_{450}SCC cDNA EcoRI inserts were subcloned into the EcoRI site of pTZ18R (Pharmacia). Clone pGBSCC-1 (figure 2), containing the largest EcoRI insert (1.4 kb), derived from the clone $\lambda\text{mbdagt11}$ SCC-54 was further analyzed by restriction enzyme mapping and sequencing.

The sequence data revealed that the pGBSCC-1 EcoRI insert was identical with the nucleotide sequence of SCC cDNA between positions 251 and 1824 on the P_{450}SCC cDNA map as described by Morohashi et al.

The remaining 5'- P_{450}SCC cDNA nucleotides were synthetically derived by cloning a 177 bp Pst/HindIII fragment into the appropriate sites of pTZ18R, resulting in the pTZ/syn lead as shown in figure 3, containing besides the nucleotides coding for the mature P_{450}SCC protein from position 188 to 273 as published by Morohashi et al., additional restrictive sites for ScaI, AvrII and StuI without affecting the predicted amino acid sequence of the P_{450}SCC protein.

The full-length P_{450} SCCcDNA was constructed by molecular cloning in E.coli JM101 (ATCC 33876) of a ligation mixture containing the 1372 bp HindIII/KpnI pGBSCC-1 fragment, the 177 bp Pst/HindIII pTZ/syn lead fragment and pTZ19R DNA digested with PstI and KpnI. The resulting plasmid, pGBSCC-2, containing all nucleotide sequences encoding the mature bovine P_{450} side chain cleavage protein is shown in figure 4.

Example 2

Construction, transformation and expression of P_{450} SCC in the bacterial host Bacillus subtilis

To derive expression of cytochrome P_{450} SCC in a Bacillus host, P_{450} SCCcDNA sequences were transferred to an E.coli/Bacillus shuttle vector pBHA-1.

Figure 5 shows the nucleotide sequence of the shuttle plasmid pBHA-1. The plasmid consists of positions 11-105 and 121-215: bacteriophage FD terminator (double); positions 221-307: a part of plasmid pBR322 (viz. positions 2069-2153); positions 313-768: bacteriophage F1, origin of replication (viz. positions 5482-5943); positions 772-2571: part of plasmid pBR322, viz. the origin of replication and the β -lactamase gene; positions 2572-2685: transposon TN903, complete genome; positions 2719-2772: tryptophan terminator (double); positions 2773-3729: transposon Tn9, the

chloramphenicolacetyl transferase gene. The nucleotides at position 3005 (A), 3038 (C), 3302 (A) and 3409 (A) differed from the wild type cat coding sequence. These mutations were introduced to eliminate the NcoI, BalI, EcoRI and PvuII sites: positions 3730-3804: multiple cloning site; positions 3807-7264: part of plasmid pUB110 containing the Bacillus "HpaII" promoter, the replication function and kanamycin resistance gene (EcoRI-PvuII fragment) (McKenzie et al., Plasmid, Vol. 15, pp 93-103, 1986 and McKenzie et al., Plasmid, Vol. 17, pp. 83-85, 1987); positions 7267-7331: multiple cloning site. The fragments were put together by known cloning techniques, e.g. filling in of sticky ends with Klenow, adapter cloning, etc. All data were derived from Genbank^R, National Nucleic Acid Sequence Data Bank, NIH, USA.

pGBSCC-3 was derived by molecular cloning in E.coli JM101 of the KpnI/SphI P₄₅₀SCCcdNA insert of pGBSCC-2 (described in Example 1) into the appropriate sites in pBHA-1 as indicated in figure 6.

By molecular cloning in E.coli JM101, the methionine initiation codon was introduced by exchanging the StuI/SphI fragment in pGBSCC-3 by a synthetically derived SphI/StuI fragment.

SPH 1

STU 1

CATATGATCAGTACTAAGACCCCTAGG

GTACGTATACTAGTCATGATTCTGGGGATCC

NDE 1

containing an NdeI site at the ATG initiation codon. The obtained plasmid pGBSCC-4 is shown in figure 7. The "Hpa II" Bacillus promoter was introduced upstream of P₄₅₀SCCcdNA sequences by digestion of the E.coli part of the shuttle plasmid by agarose gel electrophoresis and subsequent religation and transformation into Bacillus subtilis 1A40 (BGSC 1A40) competent cells. Neomycin resistant colonies were analyzed and the plasmid pGBSCC-5 (figure 8) was obtained. Expression of bovine P₄₅₀SCC was studied by preparing a cellular protein fraction of an overnight culture at 37°C in TSB medium (Gibco) containing 10 µg/ml of neomycin. Cells of 100 µl of culture, containing approximately 5.10⁶ cells, were harvested by centrifugation and resuspended in 10 mM Tris/HCl pH 7.5. Lysis was performed by adding lysozyme (1 mg/ml) and incubation during 15 minutes at 37°C. After treatment with 0.2 mg DNase/ml during 5 minutes at 37°C, the mixture was adjusted to 1x SB buffer, as described by Laemmli, Nature, Vol. 227, pp. 680-685, 1970, in a final volume of 200 µl. After heating for 5 minutes at 100°C, 15 µl of the mixture was subjected to a 7.5% of SDS/polyacrylamide gel electrophoresis. As shown in figure 9 (lane C), a 53 kDa band could be detected after immunoblotting of the gel probed with P₄₅₀SCC specific antibodies. Specific bovine P₄₅₀SCC antibodies were obtained by immunization of rabbits with purified P₄₅₀SCC protein isolated from bovine adrenal cortex tissue.

Example 3

Expression of P₄₅₀SCC in the bacterial host Bacillus licheniformis

Expression of bovine P₄₅₀SCC in B.licheniformis was performed by transformation plasmid pGBSCC-5 into the appropriate host strain B.licheniformis T5 (CBS 470.83). A cellular protein fraction prepared as described in Example 2, from an overnight culture at 37°C in Trypton Soy Broth (TSB) medium (Oxoid) containing 10 µg/ml of neomycin, was analyzed by SDS/PAGE and Western-blotting. As shown in figure 9 (lane f), a 53 kDa sized protein band was visualized after incubation of the nitrocellulose filter with antibodies specific for bovine P₄₅₀SCC. One transformant, SCC-201, was further analyzed for in vivo activity of P₄₅₀SCC (see Example 11).

Example 4

Expression of P₄₅₀SCC in the bacterial host Escherichia coli

(a) Construction of the expression cassette

To derive a suitable expression vector in the host E.coli for bovine P₄₅₀SCC, pTZ18R was mutated by site-directed mutagenesis as described by Zoller et al. (Methods in Enzymology, Vol. 100, pp. 468-500, 1983); Zoller et al. (Methods in Enzymology, Vol. 154, 329-350, 1987) and Kramer et al. (Methods in Enzymology, Vol. 154, pp. 350-367, 1987). Plasmids and strains for in vitro mutagenesis experiments were obtained from Pharmacia Inc.

A synthetic derived oligomer with the sequence:

5'-CAG GAA ACA CAT ATG ACC ATG ATT-3'

NdeI

was used to create an NdeI restriction site at the ATG initiation codon of the lac Z gene in pTZ18R. The resulting plasmid pTZ18RN was digested with NdeI and KpnI and the NdeI/KpnI DNA fragment of pGBSCC-4 containing the full-length SCCcDNA, was inserted by molecular cloning as indicated in figure 10. The transcription of P₄₅₀SCCcDNA sequences in the derived plasmid pGBSCC-17 will be driven by the E.coli lac-promoter.

(b) Expression of P₄₅₀SCC in the host E.coli JM101

pGBSCC-17 was introduced into E.coli JM101 competent cells by selecting ampicillin resistant colonies. Expression of cytochrome P₄₅₀SCC was studied by preparing a cellular protein fraction (described in Example 2) of transformants SCC-301 and 302 from an overnight culture at 37°C in 2xTY medium (containing per liter of de-ionized water: Bacto tryptone (Difco), 16 g; yeast extract (Difco), 10 g and NaCl, 5 g) containing 50 µg/ml of ampicillin.

Protein fractions were analyzed by SDS/PAGE stained with Coomassie brilliant blue (figure 11A) or by Western-blot and probed with antibodies specific for bovine P₄₅₀SCC (figure 11B). Both analyses show a protein of the expected length (figure 11A, lanes 1 and 2 and in figure 11B, lanes 3 and 4) for the transformants

SCC-301 and SCC-302, resp., which is absent in the E.coli JM101 control strain (figure 11A, lane 3 and figure 11B, lane 2).

Example 5

Construction, transformation and expression of P_{450} SCC in the yeast Kluyveromyces lactis

(a) Introduction of the geneticin resistance marker in pUC19

A DNA fragment comprising the Tn5 gene (Reiss et al., EMBO J., Vol. 3, pp. 3317-3322, 1984) conferring resistance to geneticin under the direction of the alcohol dehydrogenase I (ADHI) promoter from S.cerevisiae, similar to that described by Bennetzen et al. (J. Biol. Chem., Vol. 257, pp. 3018-3025, 1982) was inserted into SmaI site of pUC19 (Yanisch-Perron et al., Gene., Vol. 33, pp. 103-119, 1985). The obtained plasmid, pUCG418, is shown in figure 12.

E.coli containing pUCG418 was deposited at Centraal Bureau voor Schimmelcultures under CBS 872.87.

(b) Construction of the expression cassette

A vector was constructed, comprising pUCG418 (for description see Example 5(a)) cut with XbaI and HindIII, the XbaI-SalI fragment from pGB901 containing the lactase promoter (see van den Berg et al., Continuation-in-part of US patent application Serial No. 572,414: Kluyveromyces as a host strain) and synthetic DNA

comprising part of the 3' noncoding region of the lactase gene of K.lactis. This plasmid, pGB950, is depicted in figure 13. pGB950 was cut with SalI and XhoI and synthetic DNA was inserted:

SAL 1

STU 1

XHO 1

TCGACAAAAATGATCAGTACTAAGACTCCTAGGCCTATCGATTC

GTTTTTACTAGTCATGATTCTGAGGATCCGGATAGCTAAGAGCT

resulting in plasmid pGBSCC-6 as shown in figure 13.

The StuI-EcoRI fragment from pGBSCC-2 (see Example 1) containing the P₄₅₀SCC coding region was isolated and the sticky end was filled in, using Klenow DNA polymerase. This fragment was inserted into pGBSCC-6 cut with StuI. The plasmid containing the fragment in the correct orientation was called pGBSCC-7 (see figure 14).

(c) Transformation of K.lactis

K.lactis strain CBS 2360 was grown in 100 ml of YEPD-medium (1% yeast extract, 2% peptone, 2% glucose-monohydrate) containing 2.5 ml of 6.7% (w/w) yeast nitrogen base (Difco laboratories) solution to an OD₆₁₀ of about 7. From 10 ml of the culture, the cells were collected by centrifugation, washed with TE-buffer (10-mM Tris-HCl pH 7.5; 0.1 mM EDTA) and resuspended in 1 ml of TE-buffer. An equal volume of 0.2 M lithium acetate was added and the mixture was incubated for 1 hour at 30°C in a shaking waterbath.

15 μ g of pGBSCC-7 was cut at the unique SacII site in the lactase promoter, ethanol precipitated and resuspended in 15 μ l of TE-buffer. This DNA preparation was added to 100 μ l of the pre-incubated cells and the incubation was prolonged for 30 minutes. Then, an equal volume of 70% PEG 4000 was added and the mixture was incubated for 1 hour at the same temperature, followed by a heat shock of 5 minutes at 42°C. Then, 1 ml of YEPD-medium was added and the cells were incubated for 90 minutes in a shaking waterbath of 30°C. Finally, the cells were collected by centrifugation, resuspended in 300 μ l of YEPD and spread on agar plates containing 15 ml of YEPD agar with 300 μ g/ml of geneticin and were overlayered 1 hour before use with 15 ml of YEPD-agar without G418. Colonies were grown for 3 days at 30°C.

(d) Analysis of the transformants

Transformants and the control strain CBS 2360 were grown in YEPD medium for about 64 hours at 30°C. The cells were collected by centrifugation, resuspended in a physiological salt solution of an OD₆₁₀ of 300 and disrupted by shaking with glass beads for 3 minutes on a Vortex shaker at maximum speed. Cell debris was removed by centrifugation for 10 minutes at 4500 rpm in a Haraeus Christ minifuge GL. From the supernatants, 40 μ l samples were taken for analysis on immunoblots (see figure 15A, lane 3 and figure 15B, lane 4).

The results show that a protein of the expected length is

expressed in K.lactis cells transformed with pGBSCC-7. The transformant was denoted as K.lactis SCC-101.

Example 6

Construction, transformation and expression of P_{450} SCC in the yeast Saccharomyces cerevisiae

(a) Construction of the expression cassette

To delete the lactase promoter, pGB950 (see Example 4(b)) was cut with XbaI and SalI and the sticky ends were filled in using Klenow DNA polymerase and subsequently ligated. In the resulting plasmid, pGBSCC-8, the XbaI-site was destroyed, but the SalI site was maintained.

The SalI-fragment from pGB161 (see J.A. van den Berg et al., European Patent No. 96,430) containing the isocytochrome CI (cyc 1) promoter from S.cerevisiae was isolated and partially digested with XhoI. The 670 bp XhoI-SalI fragment was isolated and cloned into the SalI-site of pGBSCC-8. In the selected plasmid, pGBSCC-9, the SalI-site between the cyc 1 promoter and the 3' noncoding region of the lactase gene was maintained (figure 16) (HindIII partially digested).

The SalI-HindIII fragment from pGBSCC-7, containing the P_{450} SCC coding region was inserted in pGBSCC-9 cut with SalI and HindIII.

In the resulting plasmid, pGBSCC-10, the P₄₅₀ SCC coding region was downstream to the cyc 1 promoter (figure 17).

(b) Transformation of S.cerevisiae

S.cerevisiae strain D273-10B (ATCC 25657) was grown in 100 ml of YEPD overnight at 30°C, subsequently diluted (1:10000) in fresh medium and grown to an OD₆₁₀ of 6. The cells from 10 ml of the culture were collected by centrifugation and suspended in 5 ml of TE-buffer. Again, the cells were collected by centrifugation, suspended in 1 ml of the TE-buffer and 1 ml of 0.2 M lithium acetate was added. The cells were incubated for 1 hour in a shaking waterbath at 30°C. 15 µg of pGBSCC-10 were cut at the unique MluI-site in the cyc 1 promoter, ethanol precipitated and resuspended in 15 µl of TE. This DNA preparation was added to 100 µl of the pre-incubated yeast cells and incubated (shaking) for 30 minutes at 30°C. After addition of 115 µl of a 70% PEG4000 solution, the incubation was prolonged 60 minutes without shaking. Subsequently, a heat shock of 5 minutes at 42°C was given to the cells and 1 ml of YEPD medium was added, followed by an one and one-half hour incubation at 30°C in a shaking waterbath. Finally, the cells were collected by centrifugation, resuspended in 30 µl of YEPD and spread on YEPD agar plates containing geneticin (300 µg/ml). Colonies were grown for three days at 30°C.

(c) Analysis of the transformants

Transformants and the control strain were grown in YEPL-medium

(1% yeast extract, 2% bactopectone, 3.48% K_2HPO_4 and 2.2% of a 90% L-(+)-lactic acid solution; before sterilization, the pH was adjusted to 6.0 using a 25% ammonia solution) for 64 hours at 30°C. Further analysis was done as described in Example 5(d). The immunoblot-analysis demonstrated the expression of $P_{450}SCC$ in S.cerevisiae (figure 15A, lane 1).

Example 7

Construction, transformation and expression of pre- $P_{450}SCC$ encoding DNA in the yeast Kluyveromyces lactis

(a) Construction of the expression cassette

Plasmid pGB950 (see Example 5(b)) was cut with SalI and XhoI and synthetic DNA was inserted:

```

SAL  I
TCGACAAAAATGTTCCCTCGAGCTTTGGCAATTGAGATCCGCITTCGTTAAGGCTTGTCC
GTTTTTACAACCGAGCTCCAAACGGTAACCTCTAGCGGAAACCAATTCGGAACAGG

ACCAATCTTGTCCACTGTTGGTGAAGCTTGGGGTCACCACAGAGTTGGTACTGGTGAAGG
TCGTTAGAACAGGTGACAACCACTTCCAACCCAGTGCTGTCTCAAQCATGACCACTTCC

                                STU  I      XHO  I
TCCTGGTATCAGTACTAAGACTCCTAGCCCTATCCATTG
ACGACCATAGTCAATGATTCTCAGGATCCGGATAGCTAAGAGCT

```

resulting in plasmid pGBSCC-11 (figure 18). Analogous as described in Example 5(b), the $P_{450}SCC$ coding region of pGBSCC-2 was inserted into pGBSCC-11 cut with StuI. The plasmid containing the

fragment in the correct orientation was called pGBSCC-12 (figure 18).

(b) Transformation of K.lactis and analysis of the transformants

Transformation of K.lactis with pGBSCC-12 was performed as described in Example 5(c). The transformants were analyzed as described in Example 5(d). The analysis demonstrated the production of P₄₅₀SCC by K.lactis (figure 15B, lane 3).

Example 8

Construction, transformation and expression of pre-P₄₅₀SCC encoding DNA in the yeast Saccharomyces cerevisiae

(a) Construction of the expression cassette

The SalI-HindIII (HindIII partially digested) fragment from pGBSCC-12 containing the pre-P₄₅₀SCC coding region was inserted in pGBSCC-9 cut with SalI and HindIII. The resulting plasmid was called pGBSCC-13 (figure 19).

(b) Transformation of S.cerevisiae and analysis of the transformants

S.cerevisiae strain D273-10B was transformed with pGBSCC-13 as described in Example 6(b). The transformants were analyzed as described in Example 5(c). The result, shown in figure 15C (lane 3), demonstrated the expression of P₄₅₀SCC by S.cerevisiae. One

transformant, SCC-105, was further analyzed for in vitro activity of P₄₅₀SCC (see Example 12).

Example 9

Construction, transformation and expression in Kluyveromyces lactis of P₄₅₀SCC sequences fused to the pre-region of cytochrome oxidase VI from Saccharomyces cerevisiae

(a) Construction of the expression cassette

Plasmid pGB950 (see Example 6(b)) was cut with SalI and XhoI and synthetic DNA was inserted:

```
SAL  I
TCGACAAAAATGTTCTCTCGAGCTATCTTCAGAAACCCAGTTATCAACAGAACTTTGTT
CTTTTACAAACAGAGCTCGATAGAACTCTTTGGGTCAATAGTTGTCTTGAAACAA

GACAGCTAGACCAGGTGCTTACCACGCTACTAGATTGACTAAGAACACTTTCATCCAATC
CTCTCGATCTCGTCCACGAATGGTCCGATCATCTAACTGATTCCTTGTAAGTAGCTTAG

                                STU  1      XHO  1
CAGAAAGTACATCAGTACTAAGACTCCTTGGCCTATCGATTCT
GTCTTTCATCTAGTCATGATTCCTGAGGATCCCGATACCTAAGAGCT
```

resulting in plasmid pGBSCC-14.

The amino acid sequence from the cytochrome oxidase VI (COX VI) pre-sequence was taken from the article of Wright et al. (J. Biol. Chem., Vol. 259, pp. 15401-15407, 1984). The synthetic DNA was designed, using preferred yeast codons. The P₄₅₀SCC coding region of pGBSCC-2 was inserted into pGBSCC-14 cut with StuI,

similarly as described in Example 5(b). The plasmid containing the P₄₅₀SCC coding sequence in frame with the COX VI pre-sequence was called pGBSCC-15 (figure 20).

(b) Transformation of K.lactis and analysis of the transformants

Transformation of K.lactis with pGBSCC-15 was performed as described in Example 5(c). The transformants were analyzed as described in Example 5(d). The result (figure 15B, lane 2) shows that P₄₅₀SCC was expressed.

Example 10

Construction, transformation and expression in Saccharomyces cerevisiae of P₄₅₀SCC sequences fused to the pre-region of cytochrome oxidase VI from Saccharomyces cerevisiae

(a) Construction of the expression cassette

The SalI-HindIII (HindIII partially digested) fragment from pGBSCC-15, containing the coding region for P₄₅₀SCC fused to the COX VI pre-sequence, was inserted in pGBSCC-9 cut with SalI and HindIII. The resulting plasmid was called pGBSCC-16 (figure 21).

(b) Transformation of S.cerevisiae and analysis of the transformants

S.cerevisiae strain D273-10B was transformed with pGBSCC-16 as described in Example 6(b). The transformants were analyzed as

described in Example 6(c). The result, shown in figure 15C (lane 2), demonstrated the expression of P_{450} SCC by S.cerevisiae.

Example 11

In vivo activity of P_{450} SCC in Bacillus licheniformis SCC-201

B.licheniformis SCC-201 was obtained as described in Example 3 and the organism was inoculated in 100 ml of medium A. Medium A consisted of:

Calcium chloride-hexahydrate	1 g
Ammonium sulfate	5 g
Magnesium chloride-hexahydrate	2.25 g
Manganese sulfate-tetrahydrate	20 mg
Cobalt chloride-hexahydrate	1 mg
Citric acid-monohydrate	1.65 g
Distilled water	600 ml
Trace elements stock solution	1 ml
Antifoam (SAG 5093)	0.5 mg

Trace elements stock solution contained per l of distilled water:

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.75 g
H_3BO_3	0.60 g
KI	0.30 g
$\text{FeSO}_4 (\text{NH}_4)_2 \text{SO}_4 \cdot 2\text{H}_2\text{O}$	27 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	5 g
Citric acid. H_2O	15 g
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.45 g
$\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$	0.60 g
H_2SO_4 (96%)	3 ml

1009494.04302

After sterilization and cooling to 30°C to complete the medium, 60 g of maltose-monohydrate dissolved in 200 ml of distilled water (sterilized 20 minutes, 120°C), 200 ml 1M of potassium phosphate buffer (pH 6.8; sterilized 20 minutes, 120°C) and 1.7 g of Yeast Nitrogen base (Difco) dissolved in 100 ml of distilled water (sterilized by membrane filtration) were added to the medium. The culture was grown for 64 hours at 37°C and subsequently 2 ml of this culture were added as inoculum to 100 ml of medium A containing 10 mg of cholesterol. Cholesterol was added as a solution containing cholesterol 10 mg; TergitolTM/ethanol (1:1, v/v), 0.75 ml and Tween 80TM, 20 µl. The culture was grown for 48 hours at 37°C, whereupon the culture was extracted with 100 ml of dichloromethane. The mixture was separated by centrifugation and the organic solvent layer was collected. The extraction procedure was repeated twice and the 3 x 100 ml of dichloromethane fractions were pooled. The dichloromethane was evaporated by vacuum distillation and the dried extract (approximately 450 mg) was analyzed for pregnenolone using a gas chromatograph-mass spectrometer combination.

GC-MS analysis.

From the dried extract, a defined amount was taken and silylated by adding a mixture of pyridine bis-(trimethyl-silyl)-trifluoroacetamide and trimethylchlorosilane. The silylated sample was analyzed by a GL-MS-DS combination (Carlo Erba MEGA 5160-Finnigan MAT 311A-Kratos DS 90) in the selected ion mode. Gas

chromatography was performed under the following conditions: injection moving needle at 300°C; column M.cpsil29 0.25 inner diameter of df 0.2 μm operated at 300°C isotherm; direct introduction into MS-source.

Samples were analyzed by monitoring ions m/z 298 from pregnenolone at a resolution of 800. From the measurements, it was clear that in the case of the host strain B.licheniformis T5, no pregnenolone could be detected (detection limit 1 picogram), whereas in the case of B.licheniformis SCC-201, production of pregnenolone easily could be monitored.

Example 12

In vivo activity of P₄₅₀SCC from Saccharomyces cerevisiae SCC-105

S.cerevisiae SCC-105 was obtained as described in Example 8 and the organism was inoculated in 100 ml of medium B. Medium B contained per liter of distilled water:

Yeast extract	10 g
Bacto Peptone (Oxoid)	20 g
Lactic acid (90%)	20 g
Dipotassium phosphate	35 g
pH = 5.5 (adjusted with ammonia, 25% w/w)	

This culture was grown for 48 hours at 30°C and subsequently, this culture was used as inoculum for a fermentor containing medium

C. Medium C consisted of:

Yeast extract	100 g
Bacto Peptone (Oxoid)	200 g
Lactic acid (90%)	220 ml
Dipotassium hydrogen phosphate	35 g
Distilled water	7800 ml

pH was adjusted at pH = 6.0 with ammonia (25%) and the fermentor including the medium was sterilized (1 hour, 120°C).

After cooling, 2.4 g of geneticin dissolved in 25 ml of distilled water were sterilized by membrane filtration and added to the medium. The inoculated mixture was grown in the stirred reactor (800 rpm) at 30°C, while sterile air was passed through the broth at a rate of 300 l/h and the pH was automatically kept at 6.0 with 4N H₂SO₄ and 5% NH₄OH (5% NH₄OH in distilled water; sterilized by membrane filtration). After 48 hours, a feed of lactic acid (90%, sterilized by membrane filtration) was started at a rate of 20 g/h. The fermentation was then resumed for 40 hours, whereupon the cells were collected by centrifugation (4,000xg, 15 minutes).

The pellet was washed with 0.9% (w/w) NaCl, followed by centrifugation (4000xg, 15 minutes); the pellet washed with phosphate buffer (50 mM, pH = 7.0) and cells were collected by centrifugation (4,000xg, 15 minutes). The pellet was taken up in phosphate buffer (50 mM, pH = 7.0) resulting in a suspension of 0.5 g wet weight/ml. This suspension was treated in a Dyno^R-mill (Willy A. Bachofen Maschinenfabrik, Basel, Schweiz). Unbroken

cells were removed by centrifugation (4,000xg, 15 minutes). The cells-free extract (2250 ml, 15-20 mg protein/ml) was stored at -20°C.

P_{450} SCC was roughly purified by the following procedure. From 50 ml of thawed cell-free extract, a rough membrane fraction was pelleted by ultracentrifugation (125,000xg, 30 minutes) and resuspended in 50 ml of a 75 mM potassium phosphate solution (pH 7.0), containing 1% of sodium cholate. This dispersion was gently stirred for 1 hour at 0°C, and subsequently centrifugated (125,000xg, 60 minutes). To the thus obtained supernatant containing solubilized membrane proteins, $(NH_4)_2SO_4$ was added (30% w/v), while the pH was kept at 7.0 by adding small amounts of NH_4OH solution (6N). The suspension was stirred for 20 minutes at 0°C, after which the fraction of precipitated proteins was collected by centrifugation (15,000xg, 10 minutes). The pellet was resuspended in 2.5 ml with 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithio-threitol and 0.1 mM EDTA. This suspension was eluted over a gel filtration column (PD10, Pharmacia), yielding 3.5 ml of a desalted protein fraction (6 mg/ml), which was assayed for P_{450} SCC activity.

P_{450} SCC activity was determined by an assay which was essentially based on a method of Doering (Methods Enzymology, Vol. 15, pp. 591-596, 1969). The assay mixture consisted of the following solutions:

10034914-042602

Solution A (natural P_{450} SCC electron donating system): a 10 mM potassium phosphate buffer (pH 7.0) containing 3 mM of EDTA, 3 mM of phenylmethanesulfonyl fluoride (PMSF), 20 μ M of adrenodoxin and 1 μ M of adrenodoxin reductase (electron carriers; both purified from bovine adrenal cortex), 1 mM of NADPH (electron donor) and 15 mM glucose-6-phosphate and 8 units/ml of glucose-6-phosphate-dehydrogenase (NADPH generating systems).

Solution B (substrate): a micellar solution of 37.5 μ M cholesterol (doubly radiolabeled with [26,27- 14 C] cholesterol (40 Ci/mol) and [7 α - 3 H] cholesterol (400 Ci/mol)) in 10% (v/v) TergitolTM NP40/ethanol (1:1, v/v).

The assay was started by mixing 75 μ l of solution A with 50 μ l of solution B and 125 μ l of the roughly purified P_{450} SCC fraction (or buffer as reference). The mixture was stirred gently at 30°C. Samples (50 μ l) were drawn after 0, 30 and 180 minutes and diluted with 100 μ l of water. Methanol (100 μ l) and chloroform (150 μ l) were added to the diluted sample. After extraction and centrifugation (5,000xg, 2 minutes), the chloroform layer was collected and dried. The dry residue was dissolved in 50 μ l of acetone containing 0.5 mg of a steroid mixture (cholesterol, pregnenolone and progesterone (1:1:1, w/w/w)) and subsequently, 110 μ l of concentrated formic acid were added. The suspension was heated for 15 minutes at 120°C. Then, the 14 C/ 3 H ratio was determined by double label liquid scintillation counting. This

ratio is a direct measure for the side chain cleavage reaction because the ^{14}C -labeled side chain was evaporated from the mixture as isocaproic acid during the heating procedure.

Using this assay, it was found that the P_{450}SCC fraction, roughly purified from S.cerevisiae SCC-105, showed side chain cleavage activity. During 3 hours of incubation, 45% of the cholesterol had been converted. By means of thin layer chromatography, the reaction product was identified as pregnenolone.

Example 13

Molecular cloning of a full-length cDNA encoding the bovine cytochrome P_{450} steroid 17α -hydroxylase ($\text{P}_{450}17\alpha$).

Approximately 10^6 pfu's of the bovine adrenal cortex cDNA library described in Example 1 were selected for $\text{P}_{450}17\alpha$ cDNA sequences by screening with two ^{32}P -end labeled synthetic oligomers specific for P_{450} cDNA. Oligomer 17α -1 (5'-AGT GGC CAC TTT GGG ACG CCC AGA GAA TTC-3') and oligomer 17α -2 (5'-GAG GCT CCT GGG GTA CTT GGC ACC AGA GTG CTT GGT-3') are complementary to the bovine P_{450}SCC cDNA sequences as described by Zuber et al. (J. Biol. Chem., Vol. 261, pp. 2475-2482, 1986) from position 349 to 320 and 139 to 104, respectively.

Selection with oligomer 17 α -1 revealed \pm 1500 hybridizing pfu's. Several hybridizing pfu's were selected, purified and scaled up for preparative phage DNA isolation. The EcoRI site inserts of the recombinant lambda-gt11 DNA's were subcloned in the EcoRI site of pTZ18R. One clone, pGB17 α -1, was further characterized by restriction endonuclease mapping and DNA-sequencing. Plasmid pGB17 α -1 contained an 1.4 kb EcoRI insert complementary to the 3' part of P₄₅₀17 α from the EcoRI site at position 320 to the polyadenylation site at position 1721 as described by Zuber et al. A map of pGB17 α -1 is shown in figure 22A.

Eight hybridizing pfu's were obtained by selecting the cDNA library with oligomer 17 α -2. After purification, upscaling of recombinant phages and isolation of rec lambdagt11 DNA's, EcoRI inserts were subcloned in the EcoRI site of pTZ18R. EcoRI inserts varied in length from 270 bp to 1.5 kbp. Only one clone, pGB17 α -2 containing a 345 bp EcoRI-fragment, was further investigated by nucleotide sequencing and compared with the published P₄₅₀17 α cDNA sequence data by Zuber et al. As shown in figure 22 B, the P₄₅₀17 α cDNA sequence in pGB17 α -2 started 72 bp upstream the predicted AUG start codon at position 47 and showed complete homology with the 5' part of P₄₅₀17 α cDNA till the EcoRI site at position 320 as described by Zuber et al.

A full-length bovine P₄₅₀17 α cDNA was constructed by molecular

cloning in E.coli JM101 of a ligation mixture containing a partial EcoRI digest of pGB17 α -1 and the 345 bp EcoRI fragment of pGB17 α -2. The obtained clone pGB17 α -3 contained a full-length bovine P₄₅₀17 α cDNA and is shown in figure 22C.

Example 14

Construction and transformation of a full-length P₄₅₀17 α c-DNA clone into the yeast Kluyveromyces lactis

(a) Construction of the expression vector

To derive a suitable expression vector in yeast hosts for bovine P₄₅₀17 α , pGB17 α -3 was mutated by site-directed mutagenesis as described by Zoller et al, (Methods in Enzymology, Vol. 100, pp. 468-500, 1983); Zoller et al. (Methods in Enzymology, Vol. 154, 329-350, 1987) and Kramer et al. (Methods in Enzymology Vol. 154, pp. 350-367, 1987). Plasmids and strains for in vitro mutagenesis experiments were obtained from Pharmacia Inc.

- As indicated in figure 23, 9 bp just upstream the ATG initiation codon were changed to obtain a SalI restriction site and optimal yeast translation signals using the synthetic oligomer 17 α -3

SAL__1
5'-TCTTTGCTCCTGACTGCTGCCAGTCGACAAAAATGTCGCTGCTC-3'

10084874-043620940-489007

The resulting plasmid, pGB17 α -4, was digested with SalI and SmaI and the DNA-fragment containing the full length P₄₅₀17 α cDNA was separated by electrophoresis, isolated and transferred by molecular cloning in E.coli JM101 into the pGB950 vector (see Example 5) which was first digested with XhoI, sticky ends filled in with Klenow DNA polymerase and subsequently digested with SalI, resulting in the plasmid pGB17 α -5 as depicted in figure 24.

(b) Transformation of K.lactis

15 μ g of pGB17 α -5, cut at the unique SacII site in the lactase promoter, was used to transform K.lactis strain CBS 2360 as indicated in Example 5. Transformants were analyzed for the presence of integrated pGB17 α -5 sequences in the host genome by southern analysis. One transformant, 17 α -101 containing at least three copies of pGB17 α -5 in the genomic host DNA, was further analyzed for in vivo activity of P₄₅₀17 α (see Example 16).

Example 15

Construction and transformation of P₄₅₀17 α in the bacterial hosts Bacillus subtilis and Bacillus licheniformis

(a) Construction of the expression vector

To derive a suitable expression vector in Bacillus hosts for bovine P₄₅₀17 α , pGB17 α -3 was mutated by site-directed mutagenesis as described in Example 14. As indicated in figure 25, an NdeI

restriction site was introduced at the ATG initiation codon using the synthetic oligomer 17 α -4:

5'-GCT GCC ACC CAG ACC ATA TGT GGC TGC TCC T-3'
NdeI

The resulting plasmid, pGB17 α -6, was partially digested with EcoRI. The DNA fragment containing the full-length P₄₅₀17 α cDNA was separated by gel electrophoresis, isolated and ligated to EcoRI digested pBHA-1 DNA as shown in figure 26. The ligate was molecular cloned by transferring the ligation mixture into E.coli JM101 to obtain pGB17 α -7.

(b) Transformation of B.subtilis and B.licheniformis

The "HpaII" Bacillus promoter was introduced upstream of the P₄₅₀17 α cDNA sequences by digestion with pGB17 α -6 with the restriction enzyme NdeI, separation of the E.coli part of the shuttle plasmid by agarose gel electrophoresis and subsequent religation and transformation of B.subtilis 1A40 (BGSC 1A40) competent cells. Neomycin resistant colonies were analyzed and the plasmid pGB17 α -8 (figure 27) was obtained.

Transformation of the host B.licheniformis T5 (CBS 470.83) was also performed with pGB17 α -8. The plasmid remained stable in the appropriate Bacillus hosts as revealed by restriction analysis of pGB17 α -8, even after many generations.

Example 16

In vivo activity of $P_{450}17\alpha$ in Kluyveromyces lactis 17 α -101

K.lactis 17 α -101 was obtained as described in Example 14. The organism was inoculated in 100 ml of medium D. Medium D contained per liter of distilled water:

Yeast Extract (Difco)	10 g
Bacto Peptone (Oxoid)	20 g
Dextrose	20 g

After sterilization and cooling to 30°C, 2.68 g of Yeast Nitrogen Base (Difco) dissolved in 40 ml of distilled water (sterilized by membrane filtration) and 50 mg of neomycin dissolved in 1 ml of distilled water (sterilized by membrane filtration) were added to the medium. Subsequently, 50 mg of progesterone dissolved in 1.5 ml of dimethylformamide were added to 100 ml of medium. The culture was grown for 120 hours at 30°C and subsequently, 50 ml of culture broth were extracted with 50 ml of dichloromethane. The mixture was centrifugated and the organic solvent layer was separated. Dichloromethane was evaporated by vacuum distillation and the dried extract (about 200 mg) was taken up in 0.5 ml of chloroform. This extract contained 17 α -hydroxyprogesterone as shown by thin layer chromatography. The structure of the compound was confirmed by H-MNR and ¹³C-NMR. NMR analysis also showed that the ratio of 17 α -hydroxyprogesterone/progesterone in the extract was approximately 0.3.

Example 17

Molecular cloning of a full-length cDNA encoding the bovine cytochrome P₄₅₀ steroid 21-hydroxylase (P₄₅₀C21)

Approximately 10⁶ pfu's of the bovine adrenal cortex cDNA library, prepared as described in Example 1, were hybridized with a ³²P-end labeled oligo C21-1. This oligo containing the sequence 5'-GAT GAT GCT GCA GGT AAG CAG AGA GAA TTC-3' was a specific probe for the bovine P₄₅₀C21 gene located downstream the EcoRI site in the P₄₅₀C21 cDNA sequence as described by Yoshioka et al. (J. Biol. Chem., Vol. 261, pp. 4106-4109, 1986). From the screening, one hybridizing pfu was obtained. The EcoRI insert of this recombinant lambda-gt11 DNA was subcloned in the EcoRI site of pTZ18R resulting in a construct called PGBC21-1. As shown in figure 28, this plasmid contains a 1.53 kb EcoRI insert complementary to the P₄₅₀C21cDNA sequences from the EcoRI site at position 489 to the polyadenylation site as described by Yoshioka et al., as revealed by nucleotide sequencing.

To isolate the remaining 5' part (490 bp) of the P₄₅₀C21cDNA, a new bovine adrenal cortex cDNA library was prepared with the procedure described in Example 1 with only one modification. As primer for the first cDNA strand synthesis, an additional oligomer C21-2 was added. Oligomer C21-2 with the nucleotide sequence 5'-AAG CAG AGA GAA TTC-3' was positioned downstream from the EcoRI-site of P₄₅₀C21cDNA from position 504 to 490.

Screening of this cDNA library with a ^{32}P -end labeled oligomer C21-3 containing the P₄₅₀C21 specific sequence 5'-CTT CCA CCG GCC CGA TAG CAG GTG AGC GCC ACT GAG-3' (positions 72 to 37) revealed approximately 100 hybridizing pfu's. The EcoRI-insert of only one recombinant lambda-gt11 DNA was subcloned in the EcoRI-site of PTZ18R resulting in a construct called PGBC21-2. This plasmid (figure 28) contained an insert of 540 bp complementary to the P₄₅₀C21 cDNA sequences from position -50 to the EcoRI-site at position 489 as revealed by nucleotide sequencing.

Example 18

Construction of a P₄₅₀C21cDNA Bacillus expression vector and transformation to the bacterial hosts Bacillus subtilis and Bacillus licheniformis

(a) Construction of the expression vector

To construct a full-length P₄₅₀C21cDNA with flanking sequences specific for the Bacillus expression vector pBHA-1, the 5' part of the P₄₅₀C21 gene was first modified by the Polymerase Chain Reaction (PCR) method with pGBC21-2 as template and two specific P₄₅₀C21-oligomers as primers. Oligomers C21-4 (5'-CTC ACT GAT ATC CAT ATG GTC CTC GCA GGG CTG CTG-3') contained 21 nucleotides complementary to C21-sequences from positions 1 to 21 and 18 additional bases to create an EcoRV restriction site and an NdeI restriction site at the ATG initiation codon. Oligomer C21-5 (5'-AGC TCA GAA TTC CTT

CTG GAT GGT CAC-3'), was 21 bases complementary to the minus strand upstream the EcoRI-site at position 489.

The PCR was performed as described by Saiki et al (Science, Vol. 239, pp. 487-491, 1988) with minor modifications. The PCR was performed in a volume of 100 μ l containing: 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 200 μ M each dNTP, 1 μ M each C21-primer and 10 ng pGBC21-2 template. After denaturation (7' at 100°C) and addition to 2 U of Taq-polymerase (Cetus), the reaction mixture was performed to 25 amplification cycles (each: 2' at 55°C, 3' at 72°C, 1' at 94°C) in a DNA-amplifier apparatus (Perkin-Elmer). In the last cycle, the denaturation step was omitted. A schematic view of this P₄₅₀C21cDNA amplification is shown in figure 29.

The amplified fragment was digested with EcoRV and EcoRI and inserted by molecular cloning into the appropriate sites of pSP73 (Promega). The obtained plasmid was called pGBC21-3. As shown in figure 30, the 3' P₄₅₀C21-EcoRI fragment of pGBC21-1 was inserted in the right orientation into the EcoRI-site of pGBC21-3. The obtained vector pGBC21-4 was digested with EcoRV and KpnI (KpnI was situated in the multiple cloning site of pSP73) and the fragment containing the full-length P₄₅₀C21cDNA was isolated by gel electrophoresis and inserted into the appropriate sites of pBHA-1 by molecular cloning. The derived plasmid pGBC21-5 is illustrated in figure 31.

(b) Transformation of Bacillus

The "HpaII" Bacillus promoter was introduced upstream to the P₄₅₀C21cDNA gene by digestion with pGBC21-5 with the restriction enzyme NdeI, separation of the E.coli part of the shuttle plasmid by agarose gel electrophoresis and subsequent religation and transformation of B.subtilis 1 A40 (BGSC 1 A40) competent cells. Neomycin resistant colonies were analyzed to obtain pGBC21-6 (figure 32).

Transformation of the host B.licheniformis T5 (CBS 470.83) was also performed with pGBC21-6. The plasmid remained stable in both Bacillus hosts as revealed by restriction analysis.

Example 19

Construction of a P₄₅₀C21cDNA yeast expression vector and transformation to the yeast host Kluyveromyces lactis

(a) Construction of the expression vector

To derive a suitable expression vector in yeast hosts for bovine P₄₅₀C21-2, pGBC21-2 was mutated by site directed mutagenesis as described in Example 14. For the mutation, oligomer C21-6 (5'-CCT CTG CCT GGG TCG ACA AAA ATG GTC CTC GCA GGG-3') was used to create a SalI restriction site and optimal yeast translation signals upstream the ATG initiation codon as indicated in figure 33.

The SalI/EcoRI DNA fragment of derived plasmid pGBC21-7 was ligated to the 3' P₄₅₀C21-EcoRI-fragment of pGBC21-1 and inserted by molecular cloning into the appropriate sites of pSP73 as indicated in figure 34. Derived pGBC21-8 was cut with SalI and EcoRV (EcoRV site was situated in the multiple cloning site of pSP73) and the DNA fragment containing the full-length P₄₅₀C21cDNA was inserted into the yeast expression vector PGB950. Derived pGBC21-9 is depicted in figure 35.

(b) Transformation of K.lactis

15 µg of pGBC21-9 were digested with SacII and transformation of K.lactis CBS 2360 was performed as described in Example 5(c).

Example 20

Molecular cloning of a full-length cDNA encoding the bovine cytochrome P₄₅₀ steroid 11β-hydroxylase (P₄₅₀11β)

A bovine adrenal cortex cDNA library was prepared as described in Example 1 with one modification. An additional P₄₅₀11β-specific primer (oligomer 11β-1) with the nucleotide sequence 5'-GGC AGT GTG CTG ACA CGA-3' was added to the reaction mixture of the first strand cDNA synthesis. Oligomer 11β-1 was positioned just downstream to the translation stop codon from position 1530 to 1513. Nucleotide sequences and map positions of mentioned P₄₅₀11β-oligomers were all derived from the P₄₅₀11βcDNA sequence data

described by Morohashi et al. (J. Biochem., Vol. 102(3), pp. 559-568, 1987). The cDNA library was screened with a ^{32}P -labeled oligomer 11 β -2 (5'-CCG CAC CCT GGC CTT TGC CCA CAG TGC CAT-3') located at the 5' end of the P_{450} 11 β cDNA from position 36 to 1.

Screening with oligomer 11 β -2 revealed 6 hybridizing pfu's. These were further purified and analyzed with oligomer 11 β -3 (5'-CAG CTC AAA GAG AGT CAT CAG CAA GGG GAA GGC TGT-3', positions 990 to 955). Two out of six showed a positive hybridizing signal with ^{32}P -labeled oligomer 11 β -3. The EcoRI inserts in both 11 β -lambda-gt11 recombinants were subcloned into the EcoRI-site of pTZ118R. One clone with an EcoRI insert of 2.2 kb (pGB11 β -1) was further analyzed by restriction enzyme mapping and is shown in figure 36. pGB11 β -1 contained all coding P_{450} 11 β cDNA sequences as determined by Morohashi et al.

Example 21

Construction of a P_{450} C21cDNA Bacillus expression vector and transformation to the bacterial hosts Bacillus subtilis and Bacillus licheniformis

(a) Construction of the expression vector

A full-length P_{450} 11 β cDNA with modified flanking sequences to the Bacillus expression vector pBHA-1 was obtained by the PCR method (described in Example 18) with pGB11 β -1 as template and two

specific P₄₅₀11 β -oligomers as primers.

Oligomer 11 β -4 (5'-TTT GAT ATC GAA TTC CAT ATG GGC ACC AGA GGT GCT GCA GCC-3') contained 21 bases complementary to the mature P₄₅₀11 β cDNA sequence from position 72 to 93 and 21 bases to create EcoRV, EcoRI and NdeI restriction-sites and ATG initiation codon. Oligomer 11 β -5 (5'-TAA CGA TAT CCT CGA GGG TAC CTA CTG GAT GGC CCG GAA GGT-3) contained 21 bases complementary to the minus P₄₅₀11 β cDNA strand upstream the translation stop codon at position 1511 and 21 bases to create restriction-sites for EcoRV, XhoI and KpnI.

After PCR amplification with above mentioned template and P₄₅₀11 β -primers, the amplified fragment (1.45 kb) was digested with EcoRI and KpnI and inserted by molecular cloning into the Bacillus expression vector pBHA-1 cut with EcoRI and KpnI to obtain the vector pGB11 β -2 (see figure 36).

(b) Transformation of Bacillus

The "HpaII" Bacillus promoter was introduced upstream to the P₄₅₀11 β cDNA sequences by digestion of pGB11 β -2 with NdeI, separation of the E.coli part of the shuttle plasmid by agarose gel electrophoresis and subsequent religation (as described in Example 18) and transformation of B.subtilis 1A40 (BGSC 1A40) competent cells. Neomycin resistant colonies were analyzed and the plasmid, pGB11 β -3, was obtained. The derived plasmid pGB11 β -3 was also transmitted to the B.licheniformis host strain T5 (CBS 470.83).

Example 22

Construction of a $P_{450}11\beta$ cDNA yeast expression vector and transformation to the yeast host Kluyveromyces lactis

(a) Construction of the expression cassette

A full-length $P_{450}11\beta$ cDNA with modified flanking sequences to the yeast expression vector pGB950 was obtained by the PCR method (described in Example 18) with pGB11 β -1 as template and two specific $P_{450}11\beta$ -oligomers as primers.

Oligomer 11 β -6 (5'-CTT CAG TCG ACA AAA ATG GGC ACC AGA GGT GCT GCA GCC-3') contained 21 bases complementary to the mature $P_{450}11\beta$ cDNA sequence from position 72 to 93 and 18 additional bases to create a SalI restriction site, an optimal yeast translation signal and an ATG initiation codon. Oligomer 11 β -5 is described in Example 21(a). After PCR amplification with the above mentioned template and $P_{450}11\beta$ -primers, the amplified fragment (1.45 kb), was digested with SalI and XhoI and inserted by molecular cloning into the yeast expression vector pGB950 cut with SalI to obtain the vector pGB11 β -4 (figure 37).

(b) Transformation of K.lactis

15 μ g of pGB11 β -4 were cut at the unique SacII site in the lactase promoter and transformation of K.lactis CBS 2360 was performed as described in Example 5(c).

Example 23

Molecular cloning of a full-length cDNA encoding the bovine adrenodoxin (ADX), and subsequent transformation and expression of ADXcDNA in the yeast of Kluyveromyces lactis

(a) Molecular cloning of ADX

A full-length ADXcDNA, with 5' and 3' flanking sequences modified to the yeast expression vector pGB950, was directly obtained from a bovine adrenal cortex mRNA/cDNA pool (for detailed description see Example 1) by amplification using the PCR method (see Example 18). For the ADXcDNA amplification, two synthetic oligomer primers were synthesized.

Oligomer ADX-1 (5'-CTT CAG TCG ACA AAA ATG AGC AGC TCA GAA GAT AAA ATA-3') contained 21 bases complementary to the 5' end of the mature ADXcDNA sequence as described by Okamura et al (Proc. Natl. Acad. Sci. USA, Vol. 82, pp. 5705-5709, 1985) from positions 173 to 194. The oligomer ADX-1 contained at the 5' end 18 additional nucleotides to create a SalI restriction site, an optimal yeast translation signal and an ATG initiation codon. The oligomer ADX-2 (5'-TGT AAG GTA CCC GGG ATC CTT ATT CTA TCT TTG AGG AGT T-3') was complementary to the 3' end of the minus strand of ADXcDNA from position 561 to 540 and contained additional nucleotides for creating restriction sites for BamHI, SmaI and KpnI.

The PCR was performed as described in Example 18 with 1 μ M of

each ADX-primers and 10 μ l of mRNA/cDNA mixture (as described in Example 1) as template. A schematic view of this ADXcDNAa amplification is shown in figure 38.

The amplified fragment contained a full-length ADXcDNA sequence with modified flankings, which was characterized by restriction-site analysis and nucleotide sequencing.

(b) Construction of the expression vector

The amplified ADX cDNA fragment was digested with SalI and SmaI and inserted by molecular cloning into the yeast expression vector pGB950 cut with SalI and EcoRV. The derived plasmid pGBADX-1 is depicted in figure 38.

(c) Transformation of K.lactis

15 μ g of pGBADX-1 were cut at the unique SacII-site in the lactase promoter and transformation of K.lactis CBS 2360 was performed as described in Example 5(c).

(d) Analysis of the transformants

Two transformants, ADX-101 and ADX-102, and the control strain CBS 2360 were selected for further analysis. The strains were grown in YEPD-medium for about 64 hours at 30°C. Total cellular protein was isolated as described in Example 5(d). From the supernatants, 8 μ l samples were taken for analysis on immunoblots (see figure 39, lanes 3, 4 and 5).

The results show that a protein of the expected length (14 kDa) was expressed in K.lactis cells transformed with pGBADX-1. The in vitro ADX-activity of transformant ADX-102 is described in Example 24.

Example 24

In vitro activity of adrenodoxin obtained from Kluyveromyces lactis ADX-102

K.lactis ADX-102, obtained as described in Example 23, and control strain K.lactis CBS 2360 were grown in 100 ml of YEPD medium (1% yeast extract, 2% peptone, 2% glucose monohydrate) containing 2.5 ml of a 6.7% (w/w) yeast nitrogen base (Difco laboratories) solution and 100 mg l⁻¹ of geneticin (G418 sulfate; Gibco Ltd.), for 56 hours at 30°C. The cells were collected by centrifugation (4,000xg, 15 minutes), resuspended in a physiological salt solution and washed with a phosphate buffer (pH 7.0, 50 mM). After centrifugation (4,000xg, 15 minutes), the pellet was resuspended in a phosphate buffer (pH 7.0, 50 mM) resulting in a suspension containing 0.5 g cell wet weight/ml. The cells were disrupted using a Braun MSK Homogenizer (6 x 15 seconds, 0.45 - 0.50 mm glass beads) and unbroken cells were removed by centrifugation (4,000xg, 15 minutes). The cell-free extracts (40 mg protein/ml) were stored at -20°C.

ADX activity, i.e. electrotransfer capacity from adrenodoxin reductase to cytochrome P_{450} SCC, in the cell-free extracts was determined by a P_{450} SCC activity assay. The assay mixture consisted of the following solutions:

Solution A (natural P_{450} SCC electron donating system with the exception of ADX): a 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM of EDTA, 2 μ M of adrenodoxin reductase (purified from bovine adrenal cortex), 1 mM of NADPH (electron donor), 15 mM glucose-6-phosphate and 16 units/ml of glucose-6-phosphate-dehydrogenase (NADPH regenerating system).

Solution B (substrate and enzyme): a micellar solution of 75 μ M of cholesterol (doubly radiolabeled with [26,27- 14 C] cholesterol (40 Ci/mol) and [7 α - 3 H] cholesterol (400 Ci/mol)) and 1.5 μ M of P_{450} SCC (purified from bovine adrenal cortex) in 10% (v/v) TergitolTM NP 40/ethanol (1:1, v/v).

The assay was started by mixing 75 μ l of solution A with 50 μ l of solution B and 125 μ l of cell-free extract or 125 μ l of a potassium phosphate buffer (50 mM, pH 7.0) containing 10 μ M ADX (purified from bovine adrenal cortex). The mixture was stirred gently at 30°C. Samples were drawn after 15 minutes of incubation and diluted with 100 μ l of water. From a sample, substrate and product(s) were extracted with 100 μ l of methanol and 150 μ l of chloroform. After centrifugation (5,000xg, 2 minutes), the

chloroform layer was collected and dried. The dry residue was dissolved in 50 μ l of acetone containing 0.5 mg of a steroid mixture (cholesterol, pregnenolone and progesterone (1:1:1, w/w/w)) and subsequently 110 μ l of concentrated formic acid were added. The suspension was heated for 15 minutes at 120°C and then, the $^{14}\text{C}/^3\text{H}$ ratio was determined by double label liquid scintillation counting. The ratio was a direct measure for the side chain cleavage reaction, because the ^{14}C -labeled side chain was evaporated from the mixture as isocaproic acid during the heating procedure.

Using this assay, ADX electron carrier activity could easily be demonstrated in the cell-free extract of K.lactis ADX-102. In the assays with cell-free extract of K.lactis ADX-102 or with purified ADX, the side chain of the cholesterol was cleaved within 15 minutes in a yield of 50%, whereas in the assay with cell-free extract of the control K.lactis CBS 2360, no side chain cleavage could be detected.

Example 25

Molecular cloning and construction of a full-length cDNA encoding the bovine adrenodoxin oxidoreductase (ADR), and subsequent transformation of ADRcDNA in the yeast Kluyveromyces lactis

(a) Molecular cloning of adrenodoxin oxidoreductase

A bovine adrenal cortex cDNA library was prepared as described in Example 1 with one modification. An additional ADR-specific

primer (oligomer ADR-1) with the nucleotide sequence 5'-GGC TGG GAT CTA GGC-3' was added to the reaction mixture of the first strand cDNA synthesis. Oligomer ADR-1 was located just downstream to the translation stop codon from position 1494 to 1480. Nucleotide sequences and map positions of mentioned ADR-oligomers were all derived from the ADRcDNA sequence data described by Nonaka et al, Biochem. Biophys. Res. Comm., Vol. 145(3), pp. 1239-1247, 1987). The obtained cDNA library was screened with a ³²P-labeled oligomer ADR-2 (5'-CAC CAC ACA GAT CTG GGG GGT CTG CTC CTG TGG GGA-3').

4 hybridizing pfu's were identified and subsequently purified. However, only 1 pfu showed also a positive signal with oligomer ADR-3 (5'-TTC CAT CAG CCG CTT CCT CGG GCG AGC GGC CTC CCT-3'), which was located in the middle of the ADRcDNA (position 840 to 805). The ADRcDNA insert (approx. 2 kb) was molecular cloned into the EcoRI-site of pTZ18R. The obtained plasmid, pGBADR-1, contained a full-length ADRcDNA as revealed by restriction enzyme mapping and nucleotide sequencing. The physical map of pGBADR-1 is illustrated in figure 40.

(b) Construction of the expression cassette

A full-length ADR cDNA with modified flanking sequences to the yeast expression vector, pGB950, was obtained by the PCR method (see Example 18) with pGBADR-1 as template and two specific ADR-oligomers as primers. Oligomer ADR-4 ((5'-CGA GTG TCG ACA AAA ATG TCC ACA CAG GAG CAG ACC-3'), contained 18 bases complementary to

the mature ADRcDNA sequences from position 96 to 114 and 18 bases to introduce a SalI restriction site, an optimal yeast translation signal, and an ATG initiation codon.

Oligomer ADR-5 (5'-CGT GCT CGA GGT ACC TCA GTG CCC CAG CAG CCG CAG-3') contained 18 bases complementary to the minus strand of ADRcDNA upstream to the translation stop codon at position 1479 and 15 bases to create KpnI and XhoI restriction sites for molecular cloning in various expression vectors.

After amplification with the above mentioned template and ADR primers, the amplified fragment (1.4 kb) was digested with SalI and XhoI and inserted by molecular cloning into the yeast expression vector pGB950 cut with SalI and XhoI. The derived plasmid, pGBADR-2, is illustrated in figure 40.

(c) Transformation of K.lactis

15 µg of pGBADR-2 was cut at the unique SacII-site in the lactase promoter and transformation of K.lactis CBS 2360 was performed as described in Example 5(c).

Example 26

Molecular cloning of a full-length cDNA encoding bovine NADPH-cytochrome P₄₅₀ reductase (RED)

The bovine adrenal cortex cDNA library described in Example 1 was screened with a ³²P-labeled synthetic oligomer 5'-TGC CAG TTC GTA GAG CAC ATT GGT GCG TGG CGG GTT AGT GAT GTC CAG GT-3', specific for a conserved amino acid region within rat-, porcine- and rabbit RED as described by Katagari et al (J. Biochem., Vol. 100, pp. 945-954, 1986) and Murakami et al. (DNA, Vol. 5, pp. 1-10, 1986).

Five hybridizing pfu's were obtained and further characterized by restriction enzyme mapping and nucleotide sequencing. A full-length REDcDNA was inserted into expression vectors and transformed to appropriate hosts as mentioned in Examples 2, 3 and 6.

Example 27

Construction, transformation and expression of an expression cassette encoding the proteins P₄₅₀SCC and ADX in the yeast Kluyveromyces lactis

(a) Construction of the expression cassette

The expression cassette pGBADX-1 (see Example 23) was digested with SacII and HindIII (partially) and sticky ends were filled in using Klenow DNA polymerase. The DNA fragment comprising a part of the lactase promoter (but still functional), the coding ADX sequence and the lactase terminator was separated and isolated by agarose-gel electrophoresis and subsequently inserted into pGBSCC-7, which was first linearized by XbaI digestion (see Example 5(b))

and sticky ends filled in using Klenow DNA polymerase. The construction was set up so that a unique restriction site (SacII) was obtained, which is necessary to transfer the plasmid to K.lactis.

This unique SacII restriction site was located in the lactase promoter sequence flanking the SCC sequence, as the SacII restriction site in the lactase promoter flanking the ADX sequence was destroyed by the fill-in reaction. The obtained expression cassette pGBSCC/ADX-1 contained the coding sequence for SCC as well as for ADX, each driven by the lactase promoter.

(b) Transformation of K.lactis

Transformation of K.lactis CBS 2360 was performed as described in Example 5(c) with 15 μ g of pGBSCC/ADX-1, linearized at the unique SacII restriction site. One transformant (SCC/ADX-101) was selected from SCC and ADX expression studies.

(c) Analysis of the transformant K.lactis SCC/ADX-101

Cellular protein fractions were prepared from cultures of the SCC/ADX-101 and the control strain CBS 2360 as described in Example 5(d) and analyzed by SDS/PAGE and Western-blotting. The blot was probed with antibodies specific for SCC and ADX, respectively. Compared to the control strain, the cellular protein fraction of transformant SCC/ADX-101 showed two additional bands of expected length (53 and 14 kDa, respectively) showing the expression of both

proteins SCC and ADX. Expression levels of both in transformant SCC/ADX-101 were comparable with levels observed in transformants expressing only one protein (for SCC see figure 15A, lane 3, and for ADX figure 39, lane 5). The in vitro SCC and ADX activity of transformant SCC/ADX-101 is described in Example 28.

Example 28

In vitro activity of P_{450} SCC and adrenodoxin obtained from Kluyveromyces lactis SCC/ADX-101

K.lactis SCC/ADX-101 obtained as described in Example 27 and control strain K.lactis SCC-101 as described in Example 5(d) were grown in 1 liter of YEPD medium (1% yeast extract, 2% peptone, 2% glucose monohydrate) containing 100 mg l^{-1} of geneticin (G418 sulfate; Gibco Ltd.), for 72 hours at 30°C. The cells were collected by centrifugation (4,000xg, 15 minutes), resuspended in a physiological salt solution and washed with a phosphate buffer (pH 7.5, 75 mM). After centrifugation (4,000xg, 15 minutes), the pellet was resuspended in a phosphate buffer (pH 7.5, 75 mM) resulting in a suspension containing 0.5 g cell wet weight/ml. The cells were disrupted using a Braun MSK Homogenizer (6 x 15 seconds, 0.45 - 0.50 mm glass beads). Unbroken cells were removed by centrifugation (4,000xg, 15 minutes).

In the cell-free extracts, the activity of the protein complex P_{450} SCC/ADX was assayed by determining the cholesterol side-chain

cleaving reaction in the presence of NADPH and ADR. The assay mixture consisted of the following solutions:

Solution A (natural P_{450} SCC electron donating system with the exception of ADX): a 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM of EDTA, 2 μ M of adrenodoxin reductase (purified from bovine adrenal cortex), 1 mM of NADPH (electron donor), 15 mM of glucose-6-phosphate and 16 units/ml of glucose-6-phosphate-dehydrogenase (NADPH regenerating system).

Solution B (substrate and enzyme): a micellar solution of 37.5 μ M of cholesterol (doubly radiolabeled with [26,27- 14 C] cholesterol (40 Ci/mol) and [7 α - 3 H] cholesterol (400 Ci/mol)) in 10% (v/v) TergitolTM NP 40/ethanol (1:1, v/v).

The assay was started by mixing 75 μ l of solution A with 50 μ l of solution B and 125 μ l of cell-free extract. The mixture was stirred gently at 30°C. Samples were drawn after 60 minutes of incubation and diluted with 100 μ l of water. From a sample, substrate and products(s) were extracted with 100 μ l of methanol and 150 μ l of chloroform. After centrifugation (5,000xg, 2 minutes), the chloroform layer was collected and dried. The dry residue was dissolved in 50 μ l of acetone containing 0.5 mg of a steroid mixture (cholesterol, pregnenolone and progesterone (1:1:1, w/w/w)) and subsequently 110 μ l of concentrated formic acid were added. The suspension was heated for 15 minutes at 120°C. Then,

the $^{14}\text{C}/^3\text{H}$ ratio was determined by double label liquid scintillation counting. The ratio was a direct measure for the side-chain cleavage reaction, because the ^{14}C -labeled side chain was evaporated from the mixture as isocaproic acid during the heating procedure.

Using this assay, cholesterol side-chain cleaving activity was demonstrated in the cell-free extract of K.lactis SCC/ADX-101, whereas the cell-free extract of K.lactis SCC-101, no activity was detectable. By means of HPLC-analysis, the reaction product produced by a cell-free extract of K.lactis SCC/ADX-101 was identified as pregnenolone.

Example 29

Construction and transformation of an expression cassette encoding bovine cytochrome P_{450} steroid 17α -hydroxylase and bovine cytochrome P_{450} steroid C21-hydroxylase in the yeast Kluyveromyces lactis

(a) Construction of the expression cassette

The expression cassette pGB17 α -5 (figure 41) described in Example 14, was digested with SacII and HindIII (partially) and sticky ends were filled in using Klenow DNA polymerase. The DNA fragment comprising a part of the lactase promoter, the sequence coding for $\text{P}_{450}17\alpha$ and the lactase terminator were separated and isolated by agarose gel electrophoresis and subsequently inserted into pGBC21-9 (figure 42) described in Example 19, which was first

linearized by XbaI digestion and sticky ends filled in using Klenow DNA polymerase. The obtained expression cassette, pGB17 α /C21-1 (figure 43), had a unique SacII restriction site because the SacII restriction site in the lactase promotor flanking the P₄₅₀17 α sequence was destroyed by the fill-in reaction.

(b) Transformation of K.lactis

Transformation of K.lactis CBS 2360 was performed as described in Example 5(c) with 15 μ g of pGB17 α /C21-1, linearized at the unique SacII restriction site. One transformant 17 α /C21-101 was further analyzed for in vivo activity of both, P₄₅₀17 α and P₄₅₀C21 (see Examples 30 and 31).

Example 30

In vitro activity of P₄₅₀17 α and P₄₅₀C21 obtained from Kluyveromyces lactis 17 α /C21-101

K.lactis 17 α /C21-101 obtained as described in Example 29, K.lactis 17 α -101 as described in Example 14 and K.lactis CBS 2360 were grown in 100 ml of medium D. Medium D contained per liter of distilled water:

Yeast extract (Difco)	10 g
Bacto Peptone (Oxoid)	20 g
Dextrose	20 g
pH = 6.5	

1006464-042602

After sterilization and cooling to 30°C, 25 mg of geneticin (G418 sulfate; Gibco Ltd.) dissolved in 1 ml of distilled water sterilized by membrane filtration was added. The cultures were grown for 72 hours at 30°C and the cells were collected by centrifugation (4,000xg, 15 minutes). The pellet was washed with phosphate buffer (50 mM, pH=7.0) and cells were collected by centrifugation (4,000xg, 15 minutes). The pellet was taken up in phosphate buffer (50 mM, pH=7.0) resulting in a suspension containing 0.5 g wet weight/ml. This suspension was disrupted by sonification (Braun labsonic 1510; 12x1 minute, 50 Watts). Unbroken cells were removed by centrifugation (12,000xg, 15 minutes).

Cell-free extracts were assayed for $P_{450}17\alpha$ activity and $P_{450}C21$ activity by determining the production of $17\alpha,21$ dihydroxyprogesterone in the presence of NADPH. The assay mixture consisted of the following solutions:

Solution A: a 50 mM potassium phosphate buffer (pH=7.0), containing 3 mM of EDTA, 2 mM of NADPH, 50 mM of glucose-6-phosphate and 16 units/ml of glucose-6-phosphate-dehydrogenase (NADPH-regenerating system).

Solution B (substrate): a micellar solution of 80 μ M of [4- 14 C] progesterone (30 Ci/mol) in 10% (v/v) TergitolTM NP 40/ethanol (1:1, v/v) in a potassium phosphate buffer (75 mM, pH=7.5).

10084844-042602

The assay was started by mixing 75 μ l of Solution A with 50 μ l of Solution B and 125 μ l of cell-free extract. The mixture was stirred gently at 30°C and 50 μ l samples were drawn after 60 minutes of incubation and added to a mixture of 100 μ l of methanol and 50 μ l of chloroform. Subsequently, 100 μ l of chloroform and 100 μ l of water were added. The chloroform layer was collected by centrifugation (5,000xg, 2 minutes) and the water/methanol layer was re-extracted with 100 μ l of chloroform. The two chloroform layers were combined and dried. The dry residue was dissolved in 100 μ l of acetonitrile/H₂O (9:1, v/v) and 50 μ l samples were eluted with acetonitrile/H₂O (58:42, v/v) using an HPLC column (Chrompack licher. 10RP18, 250x4.6 mm). In the eluate, the steroid substrate and products were detected by a flow scintillation counter and a U.V. detector. The radioactivity of the collected fractions was determined by liquid scintillation counting. Using the assay, it was found that a cell-free extract obtained from K.lactis 17 α /C21-101 produced 17 α ,21 dihydroxy progesterone, whereas cell-free extracts obtained from K.lactis 17 α -101 and K.lactis CBS 2360 did not. The main product produced by K.lactis 17 α -101 appeared to be 17 α hydroxy progesterone.

Example 31

In vivo activity of P₄₅₀17 α and P₄₅₀C21 in Kluyveromyces lactis 17 α /C21-101

K.lactis 17 α /C21-101 obtained as described in Example 29 and K.lactis CBS 2360 were inoculated in 25 ml of medium D. Medium D contained per liter of distilled water:

Yeast extract (Difco)	10 g
Bacto Peptone (Oxoid)	20 g
Dextrose	20 g
pH = 6.5	

After sterilization and cooling to 30°C, 25 mg of geneticin dissolved in 1 ml of distilled water sterilized by membrane-filtration was added to 1 liter of medium D. Then, 100 μ l of a solution containing the substrate [4-¹⁴C] progesterone were added to 25 ml of the completed medium. The substrate solution contained 800 μ l [4-¹⁴C] progesterone (8 Ci/mole) per ml in 10% (v/v) TergitolTM NP 40/ethanol (1:1, v/v). The cultures were grown at 30°C in a rotary shaker (240 rpm) and samples of 2 ml taken after 0 and 68 hours were drawn. Each sample was mixed with 2 ml of methanol. After 24 hours of extraction at 4°C, the mixtures were centrifugated (4,000xg, 15 minutes). From the obtained supernatant, samples of 200 μ l were eluted with acetonitrile/H₂O (58:42, v/v) using an HPLC column (Chrompack Lichr. 10 RP18, 250x4.6 mm).

In the eluate, the steroid substrate and products were detected. The radioactivity of the collected fractions was determined by liquid scintillation counting. One of the fractions

obtained from a culture of K.lactis 17 α /C21-101 grown for 68 hours clearly showed the presence of 17 α ,21 dihydroxyprogesterone, whereas this compound was not produced in a culture of the control strain K.lactis CBS2360.

Example 32

Construction, transformation and expression of an expression cassette encoding the human 3 β -HSDH in the yeast Saccharomyces cerevisiae

1. Generation of pUC derivatives with new polylinker sites.

M13mp19 (Yanisch-Peron, C. et al., Gene 33 (1985) p 103-119) was mutagenized using oligonucleotide

OTG2805: 5' GCGCTCAGCGGCCGCTTTCCAGTCG 3'

and a NotI site was introduced into the remaining sequence of the lacI gene (M13TG724). Then, a polylinker containing EcoRI, SnaBI and NotI sites was introduced in the EcoRI site of M13TG724 using oligonucleotides

OTG2793: 5' AATTGCGGCCGCGTACGTATG 3' and

OTG2796: 5' AATTCATACGTACGCGGCCGC 3'

However, during the cloning step, multiplication and modification of the insert occurred. The resulting M13TG7244 had the following sequence:

GAATTCATACGTACGCGGCCGCAATTGCGGCCGGTACGTATAATTCACTGGCCGT

Note that the EcoRI, SnaBI and NotI sites are underlined and that the lacZ sequence of pUC19 is in italics. M13TG7244 was digested

with EcoRI and SstI restriction enzymes and a linker was introduced using oligonucleotides

OTG2919: 5' CAACGCGTCCTAGG 3' and

OTG2920: 5' AATTCCTAGGACGCGTTGAGCT 3'

yielding M13TG7246. This linker added MluI and AvrII sites. A PvuII fragment containing the relevant restriction sites of M13TG7246 was subcloned into pUC19, yielding pTG7457 (figure 44).

pUC19 (Yanisch-Peron, C. et al., Gene 33 (1985) p 103-119) was digested with BamHI and EcoRI restriction enzymes and a polylinker was introduced using oligonucleotides

OTG2792: 5' GATCCGCAGATATCATCTAGATCCCGGGTAGAT 3',

OTG2797: 5' AGAGCTCAAGATCTACCCGGGATCTAGATGATATCTGCG 3',

OTG2794: 5' CTTGAGCTCTACGCAGCTGGTCGACACCTAGGAG 3' and

OTG2795: 5' AATTCTCCTAGGTGTGCGACCAGCTGCGT 3'

yielding pTG7453 (figure 45).

Subcloning of the terminator.

PGK terminator:

The polylinker sites between BamHI and SstI of pTG7453 were introduced into a pTG7457 derivative and the new plasmid was digested with BglII and HindIII restriction enzymes and a similarly restricted fragment containing the PGK terminator (Hitzeman, R.A. et al., Nucleic Acids Res. 10 (1982) 7791- 7808) ; Loison, G. et al., Yeast 5 (1989) p 497-507) was cloned into it. The new plasmid was termed pTG10014 (figure 46). pTG10014 was digested with ClaI

restriction enzyme and the cohesive ends filled in with the Klenow polymerase yielding pTG10015.

Subcloning of the promoters.

a) The CYC1 promoter:

The polylinker sites between BamHI and SstI of pTG7453 were introduced into a pTG7457 derivative and the new plasmid was opened by SnaBI restriction enzyme and the RsaI DraI fragment of 456 nucleotides of pEMBL8 (Dente et al., Nucleic Acids Res. 11 (1983) p 1645-1655), containing the origin of replication of phage f1, was introduced generating pTG7503. A 0.78kb BamHI HindIII fragment of pGBSCC-9, prepared in Example 6 containing the CYC1 promoter of *Saccharomyces cerevisiae*, a polylinker and the lactase terminator of *Kluyveromyces lactis*, were subcloned in pTG7503, yielding pTG10004 (figure 47). The XhoI and MluI sites of the CYC1 promoter were eliminated by site directed mutagenesis using oligonucleotide OTG4410: 5' GCGGATCTGCTCGAAGATTGCCTGCGCGTTGGGCTTGATC 3' on ssDNA of pTG10004. This yielded pTG10005. pTG10005 was digested with SalI and XhoI restriction enzymes and a MluI site was introduced using oligonucleotides OTG4433: 5' TCGACGGACGCGTGG 3' and OTG4434: 5' TCGACCACGCGTCC 3' yielding pTG10006.

b) The GAL10/CYC1 promoter:

The pYeDP1/8-2 (Cullin, C., Gene 65 (1988) p 203-217) plasmid

was opened with XhoI restriction enzyme. The cohesive ends were filled in with klenow polymerase and the plasmid was religated. In pTG10010, the GAL10/CYC1 promoter no longer contained the XhoI site and this served as a template for a PCR amplification.

2. Construction of the expression vectors.

In pTG7503, part of the remaining lacZ coding sequence was eliminated by site directed mutagenesis using oligonucleotide

OTG4431: 5' TGGCCGTCGTTTTACTCCTGCGCCTGATGCGGTAT 3'

yielding pTG7549. The LacZ promoter present in pTG7549 was deleted using oligonucleotides

OTG4470: 5' GGCCGCAAAACCAAA 3' and

OTG4471: 5' AGCTTTTGGTTTTGC 3'

which were inserted after a NotI HindIII restriction, restoring both sites. The new construct was termed pTG7553. A BamHI MluI fragment containing the CYC1 promoter of pTG10006 and the MluI HindIII fragment containing the PGK terminator of pTG10015 were ligated together. The ligation material was then added to pTG7553, previously digested with MluI and HindIII restriction enzymes. Finally, oligonucleotide

OTG4478: 5' GATCTATCGATGCGGCCGCG 3'

hybridized with oligonucleotide

OTG4479: 5' CGCGCGCGGCCGCGCATCGATA 3'

(BamHI MluI linkers containing ClaI NotI sites) was added, and ligated together. The resulting plasmid was termed pTG10031 (figure 48). The PCR amplified fragment obtained with pYeDP1/8-2 was

digested with ClaI and SalI restriction enzymes and introduced into pTG10031 digested with the same enzymes yielding pTG10033 (figure 49).

3. Construction of the basic vector

pTG3828 (Achstetter, T. et al., Gene 110 (1992) p 25-31) was digested with BglII and BamHI restriction enzymes and a polylinker segment of pPOLYIII (Lathe R. et al., Gene 57 (1987) p 193-201) covering the BglII BamHI sites was introduced. The orientation which had lost the BglII and BamHI sites was chosen (pTG10012). pTG10012 was digested with NotI and EcoRI restriction enzymes and ligated to the large EcoRI NotI fragment of pTG7549 and this generated pTG10013 (figure 50). The URA3-D fragment on this plasmid was bordered by HindIII sites.

4. Construction of the recombination vectors.

The NotI cassettes containing the CYC1 or the GAL10/CYC1 promoter and the PGK terminator were subcloned into vectors containing the URA3-d gene in both orientations, yielding recombination vectors pTG10041-pTG10042 and pTG10045-pTG10046 respectively. pTG10013 was digested with NotI restriction enzyme and the NotI fragments containing the expression block of pTG10031 were ligated into it yielding two orientations, pTG10041 (figure 51) and pTG10042 (figure 52). Similarly, pTG10045 (figure 53) and pTG10046 (figure 54) were obtained from pTG10033 and pTG10013.

5. Construction of transfer vectors

The cDNA coding for human 3BHSDH Type I, obtained from Labrie, was contained in the EcoRI site of pT7T3 vector. The coding sequence corresponded to the sequence published previously by V. Luu The et al. (1989) Mol. Endocrinol. Vol. 3, pp. 1310-1312, except that the 5' contained an additional GGG. This plasmid was modified to allow direct cloning in the expression vectors. First, a linker containing a MluI site using oligonucleotide

OTG4539 : 5' AATTGGACGCGTCC 3'

was introduced at the 3' end of the coding sequence after partial EcoRI digestion (pTG10036). The EcoRI site of pTG10036 was treated by Mung Bean Nuclease and the resultant DNA was digested by MluI restriction enzyme. On the other hand, a SalI MluI insert of 1.7 kb was cloned into the SalI MluI sites of pTG10031 (pTG10058). The SalI site of pTG10058 which carried the CYC1 promoter was filled in with Klenow polymerase and the resultant plasmid was digested with MluI restriction enzyme. Ligation of these fragments yielded pTG10064 (figure 55) which contained the coding sequence for 3BHSDH bordered by SalI and MluI restriction sites. The SalI MluI fragment from pTG10064 was subcloned into pTG10033 yielding pTG10065 (figure 56) with 3BHSDH under control of GAL10/CYC1.

6. Expression of 3BHSDH in *S. cerevisiae*

The yeast used in this study was strain W303-1B (*MAT α* , ρ^+ , *ura3-1*, *leu2-3*, *-112*, *his3-11*, *-15*, *trp1-1*, *ade2-1*, *can^R* (?), *cyr⁺* (?)) [Crivellone et al., J. Biol. Chem. 263 (1988) 14323-14333]

as a model strain. W303-1B was auxotrophic for uracil, leucine, histidine, tryptophane, adenine and resistant to canavanine.

a) Construction of a yeast- E.coli shuttle vector for the expression of 3BHSDH.

Using the recombination vectors, assembly of the desired expression plasmid via recombination *in vivo* can be accomplished. Yeast was made electrocompetent and was transformed using 100 ng of pTG10042 (restricted with SalI and MluI) and pTG10065 (restricted with NotI). After electroporation, the cells were plated on selective YNBG medium containing adenine, tryptophane, leucine and histidine but no uracil. After 3-4 days of growth, colonies were purified on the same medium. *Saccharomyces cerevisiae* recombined expression plasmid (SCREP) pTG10201 (PromCYC1 - 3B HSDH - PGKterm) was obtained in strain (W303-1B). The latter SCREP was obtained by an *in vivo* recombination between the *GAL10/CYC1* promoter and the *CYC1* promoter, reconstructing the *CYC1* promoter.

b) Detection of 3B HSDH antigen in yeast by Western blotting.
Antibodies

The rabbit anti-3B HSDH antibodies against human 3BHSDH were obtained from F. Labrie and have been described in V. Luu The et al., (1989) Mol. Endocrinol. Vol. 3, pp. 1310-1312.

Western blotting

Western blotting was done as described previously (E. Degryse

et al., (1992) Gene Vol. 118, pp. 47-53) except that anti-3 β HSDH antibodies were used.

c) Compartmentation.

Compartmentation was studied in strain W303-1B transformed with SCREP pTG10201 (CYC1 + 3 β HSDH). Cells were divided into cytosol, mitochondria and microsomes according to C. Cullin and al., Gene 65 (1988) p 203-217. Western blots and activity measurements were performed on fractions of the untransformed and two transformed strains. The control (untransformed strain) was found to be negative all over. For the other strains, most of the activity was found in the mitochondrial and microsomal fractions. These results were confirmed in the Western blot, showing the correct size of the expressed gene product.

d) In vitro activity of 3 β HSDH after induction in minimal medium and cell fractionation.

Cells were grown until an optical density between 2 and 5 at 600nm, in YNBG medium supplemented with casamino acids (0,5 %) and tryptophane, adenine, histidine and leucine. After harvest of the cells by centrifugation, microsomes were prepared according to C. Cullin and al., Gene 65 (1988) p 203-217. The 3 β HSDH activity was measured with the method of Bauer H.C. et al., J. Steroid Biochem. 33 (1989) 643-646. The microsomes were taken up in TrisHCl 50mM pH7.4, EDTA 2mM, glycerol 20% and stored at -20°C until used. The protein concentration was 3.75 mg/ml. Activity was measured in DPBS

+ BSA (0.175 ml) + pregnenolone (0.005ml; $1\mu\text{Ci} = 40\text{nmole/ml}$) and 19 μg of microsomal extract/tube. The reaction was started by the addition of NAD 10mM (0.015 μl) and lasted for 60 minutes at 37°C. Controls were used in which the reaction was stopped immediately after substrate addition to the complete reaction mixture (t=0h) or to the NAD deleted reaction mixture

1006484-046606

(t=0h-NAD).

The in vitro activity of 3 β HSDH was measured using radioactive pregnenolone. After incubation at 37°C, the bulk of the pregnenolone was precipitated with digitonin and the soluble product (progesterone) was measured by scintillation counting. A microsomal fraction of W303-1B/pTG10201 (CYC1 + 3 β HSDH) was shown to be active in the presence of NAD only.

NAD dependence of 3 β HSDH activity

	t=0h - (t=0h-NAD)	60'- (t=0h -NAD)
+ NAD	17 000	463 000
- NAD	0	0

Activity was also measured after cell fractionation (see above). Activity was measured in DPBS + BSA (0.077ml) + pregnenolone (0.002ml; 1 μ Ci=40nmole/ml) + NAD (0.010 μ l, 10mM) and 7 μ g of cytosolic extract, 9 μ g of mitochondrial extract and 6 μ g of microsomal extract/tube.

W303	net CPM/15'/ μ g
cytosol	312
mitochondria	297
microsomes	178

W303/pTG1020 (Prom CYC1 3B HSDH PGK

1 term) Percentage

cytosol	6577	9.7%
mitochondria	42308	62.7%
microsomes	18590	27.5%

The activity measurements coincided with the results of the Western blot. Activity was found in the mitochondrial and microsomal fraction. No activity was present in the cytosol nor in any fraction obtained from untransformed yeast. After a 15 minutes incubation at 37°C, the conversion of pregnenolone (0.8 μ M) into progesterone was measured for 1 μ g amounts of the following fractions: mitochondria, 60% conversion; micro-somes, 15% conversion, cytosol, 3% conversion. The reaction was not linear with time, but this point was not investigated further. The conversion of pregnenolone into progesterone was demonstrated in vivo (see below).

e) In vivo activity of 3 β HSDH

Bioconversion of pregnenolone into progesterone:

Transformed yeast cells were grown in the presence of 100 μ g/ml of pregnenolone and samples were extracted and analyzed by RP-HPLC for conversion into progesterone. The preliminary results showed the accumulation of progesterone in the culture medium of transformed yeast. 15% of pregnenolone was converted into progesterone in 2 days.

Example 33

Construction, transformation and bioconversion from an expression cassette encoding human 3 β -HSDH and bovine P450 17 α in Saccharomyces cerevisiae

1. Construction of transfer vectors for human 3 β -HSDH and bovine P₄₅₀ 17 α
 - a) Sub-cloning of the cDNA coding for the bovine P₄₅₀ 17 α :
The cDNA coding for the bovine P450 in plasmid pGB17 α -5 described in example 14 (figure 24) was reformatted on SalI MluI restriction sites by introduction of a MluI site into the XhoI site of the vector. The SalI MluI fragment was subcloned into pTG10031 (example 32, figure 48) under control of the CYC1 promoter. This vector was called pTG10058.
 - b) Sub-cloning of the human 3 β -HSDH type I :
The vector pTG 10065 with cDNA encoding human 3 β -HSDH type I

under control of the Gal10/CYC1 promoter was obtained in Example 32 (figure 56).

2. Construction of a yeast-E.coli shuttle vector for the expression of human 3B-HSDH type I and bovine P₄₅₀17 α .

Using recombination vectors, assembly of the desired expression plasmid via recombination in vivo can be accomplished. Plasmids containing CYC1_{prom}-PGK_{term} and the cDNAs for bovine P₄₅₀ 17 α or human 3B-HSDH type I were coupled with LEU2 or URA3-d as selection markers, respectively. Recombination vectors were generated containing the yeast 2 μ m; a replicon, an expression cassette with the CYC1_{prom}-PGK_{term} and different selection markers URA3-d (pTG10259) or LEU2 (pTG10260). pTG10259 is identical to the previously described recombination vector pTG10042 previously described in Example 32 (figure 52) except that the single XbaI site contained in the 2 μ m region was replaced by a XbaI site, obtained through filling in by the Klenow polymerase and religation.

The LEU2 containing recombination vector was constructed as follows. Into a plasmid containing the LEU2 gene (Genbank locus YSCLEU2, accession number J01333), HindIII sites into the HpaI site (located at position 241 were introduced in YSCLEU2, using as an adaptor oligonucleotide OTG4464 : CACAAGCTTGTG) and SalI site (at position 2213 in YSCLEU2, using as an adaptor oligonucleotide OTG4463 : TCGAGGGAAGCT). The HindIII fragment was

cloned into pTG10013 (see figure 50) digested with HindIII restriction enzyme and was treated with phosphatase. This yielded both orientations, pTG10023 and pTG10024. A NotI fragment containing the expression block of pTG10031 (figure 48) was subcloned into pTG10023 and yielded pTG10158. The orientation of the selection marker and the expression block in pTG10158 was similar to that present in pTG10042 (figure 52). pTG10158 generated pTG10260 simply by eliminating the single XbaI site contained in the 2 μ m region, as done above. The expression blocks from pTG10065 (human 3 β -HSDH type I) and pTG10058 (bovine P₄₅₀ 17 α) were introduced next, yielding the final expression plasmids, respectively pTG10261 and pTG10269.

3. Transformation of *Saccharomyces cerevisiae*

Yeast strain W303-1B was transformed using a transformation protocol described by Lauermann, Curr. Genetics, Vol. 20, pp. 1-3, (1991). Ethanol improved the transformation efficiency of intact yeast cells. The yeast cells were transformed with 1 μ g of pTG10261 + pTG10269 (no carrier DNA was utilized). After transformation and plating out on agar plates containing YNBG + casamino acids 0.01% + WAH or YNBG casamino acids 0.01% + WAHL, candidate colonies were confirmed on selective medium. Selection was done on YNBG + casamino acids 0.01% + WAH (W = Trp, A = adenine, H = His). PCR was used to confirm the simultaneous presence of the selective marker and the cDNA associated with it.

4. In vivo activity of P₄₅₀ 17 α and 3 β -HSDH in *Saccharomyces cerevisiae*

Bioconversion was measured with 100 μ g/ml of pregnenolone incubated at 30°C with cells on YNB + glycerol + WAH medium. Samples were taken after 2 days, then were extracted and analyzed by RP-HPLC. Values for 17 α -hydroxyprogesterone (17OH-PROG) and progesterone (PROG) were expressed as μ g/ml with duplicate data from different clones. The following results were obtained.

W303-1B	Product		Product	
	PROG	17OH-PROG	PROG	17OH-PROG
pTG10261 + pTG10269	1.6	3.2	0.6	4.6

The bioconversion of pregnenolone into 17 α -hydroxyprogesterone was achieved in yeast by coexpression of 3 β -HSDH and P₄₅₀ 17 α .

Example 34

In vivo activity of bovine P₄₅₀17 α in transformed Kluyveromyces lactis

Transformation of K.lactis strain CBS 2360 was performed with pGB 17 α -5 as described in Example 14 and in vivo activity was determined in the whole cells of the transformed K.lactis strain. Four independent transformants (17 α -3, 17 α -7, 17 α -10 and 17 α -11) were grown in rich medium (10 g of yeast extracts, 10g of bactopectone and 20 g of glucose/liter) for 72 hours to reach an A600 of at least 30. The cells were harvested and resuspended at 1×10^6 cells/ml (A600 = 1).

Five ml of culture were incubated in the presence of ³H-labelled progesterone (20 μ M) for 24 hours at 28°C and then were extracted with dichloromethane. RP-HPLC analysis showed that progesterone was specifically transformed into 17 α -hydroxyprogesterone. The amount of 17 α -hydroxyprogesterone was about 40% (about 23% to 58% depending on the transformant) of the substrate added (see figure 57 corresponding to 17 α -3). No androstenedione, which was the product of the C17-20-lyase activity of P₄₅₀17 α , was detected.

³H-labelled 17 α -hydroxyprogesterone was also incubated with the transformants under the conditions described above and after 24 hours of incubation, no androstenedione was detected. These

data indicate that the bovine P₄₅₀17 α expressed in K.lactis under the lactase promoter does not show any activity C17-20 lyase.

Various modifications of the products and method of the invention may be made without departing from the spirit or scope thereof and it is to be understood that the invention is intended to be limited only as defined in the appended claims.

2003-01-04 10:00:00

WHAT WE CLAIM IS:

1. An expression cassette, operable in a recombinant host, comprising a heterologous DNA coding sequence encoding a protein, which is functional, alone or in cooperation with one or more additional proteins, of catalyzing an oxidation step in the biological pathway for conversion of cholesterol into hydrocortisone, which step is selected from the group consisting of:

the conversion of cholesterol to
pregnenolone;

the conversion of pregnenolone to progesterone;

the conversion of progesterone to 17 α -hydroxy-
progesterone;

the conversion of 17 α -hydroxyprogesterone to
cortexolone;

the conversion of cortexolone to hydrocortisone, and
the corresponding control sequences effective in said
host.

2. An expression cassette of Claim 1, wherein the heterologous DNA coding sequence encodes at least two proteins which are functional of catalyzing, alone or in cooperation with one or more additional proteins, at least two oxidation steps of the group of Claim 1.

- 10084974-042602
3. An expression cassette of Claim 1, wherein it contains at least one additional heterologous DNA with its own effective control sequences, encoding a protein, which is functional, alone or in cooperation with one or more additional proteins, of catalyzing an oxidation step of the group of Claim 1.
 4. An expression cassette of Claim 1 wherein the protein is selected from the group consisting of side-chain cleaving enzyme ($P_{450}SCC$); adrenodoxin (ADX); adrenodoxin reductase (ADR); 3β -hydroxysteroid dehydrogenase/isomerase (3β -HSD); steroid- 17α -hydroxylase ($P_{450}17\alpha$); NADPH cytochrome P_{450} reductase (RED); steroid- 21 -hydroxylase ($P_{450}C21$), and steroid- 11β -hydroxylase ($P_{450}11\beta$).
 5. An expression cassette of Claim 4, wherein the heterologous DNA coding sequences originate from bovine species.
 6. An expression cassette of Claim 4 wherein the heterologous DNA encodes at least one additional protein from the group of Claim 4.
 7. An expression cassette of Claim 4 wherein it contains at least one additional heterologous DNA with its own effective control sequences encoding a protein from the group of Claim 3.

8. An expression cassette of Claim 6 wherein the heterologous DNA encodes bovine P_{450}^{SCC} and bovine ADX.
9. An expression cassette of Claim 5 wherein the heterologous DNA encodes the enzyme P_{450}^{SCC} and that the expression cassette is denoted as pGBSCC-n where n is an integer from 1 to 17.
10. An expression cassette of Claim 5 wherein the heterologous DNA encodes the enzyme $P_{450}^{17\alpha}$ and the expression cassette is denoted as pGB17 α -n where n is an integer from 1 to 5.
11. An expression cassette of Claim 5 wherein the heterologous DNA encodes the enzyme P_{450}^{C21} and the expression cassette is denoted as pGBC21-n where n is an integer from 1 to 9.
12. An expression cassette of Claim 5 wherein the heterologous DNA encodes the enzyme $P_{450}^{11\beta}$ and the expression cassette is denoted as pGB11 β -n where n is an integer from 1 to 4.
13. A recombinant host cell and progeny thereof comprising cells of micro-organisms, plants or animals and containing an expression cassette with heterologous DNA characterized in that the expression cassette is that of Claim 1.
14. A recombinant host cell and progeny thereof of Claim 13

wherein the host is a micro-organism.

15. A recombinant host cell and progeny thereof of Claim 14 wherein the host is selected from the group consisting of Saccharomyces, Kluyveromyces, Bacillus and Escherichia coli.
16. A recombinant host cell and progeny thereof of Claim 13 and containing at least two expression cassettes as defined in Claim 1.
17. A process for the preparation of an exogenous protein by a recombinant cell comprising culturing the recombinant cell in a nutrient medium under conditions enabling the protein to be formed and accumulated in the culture, wherein the recombinant cell is a recombinant host cell as defined in Claim 13.
18. A process for the preparation of a mixture of endogenous proteins by a recombinant cell in a nutrient medium under conditions enabling the enzymes to be formed and accumulated in the culture wherein the recombinant cell is a recombinant host cell as defined in Claim 16.
19. A process for selective biochemical oxidation in vitro comprising incubating the compound to be oxidized in the presence of at least one protein under conditions which

permit said oxidation and the accumulation of the oxidized compound in the culture liquid, followed by recovering the oxidized compound, characterized in that the protein or proteins have been produced by the process of Claims 17.

20. In a process for oxidizing a selected compound comprising culturing recombinant cells in the presence of said compound under conditions wherein the desired oxidation occurs and the oxidized compound accumulates in the culture liquid and recovering the oxidized compound, the improvement comprising using a recombinant host cell of Claim 13.
21. A process of Claim 20, wherein the oxidation is a step selected from the group consisting of: cleaving the side-chain of a sterol compound to pregnenolone; the conversion of pregnenolone to progesterone; the conversion of progesterone to 17 α -hydroxyprogesterone; the conversion of 17 α -hydroxyprogesterone to cortexolone and the conversion of cortexolone to hydrocortisone.
22. The process of Claim 21 wherein the oxidation is cleaving the side-chain of a sterol compound resulting in pregnenolone.
23. The process of Claim 21 wherein the oxidation is the 17 α -hydroxylation of progesterone.

24. The process of Claim 21 wherein at least two oxidations from said group are carried out on the same substrate molecule in one step.

25. A recombinant host cell and progeny thereof comprising cells of micro-organisms, plants or animals containing, besides proper control sequences, heterologous DNA coding for proteins either from the group comprising proteins which are functional, alone or in cooperation with one or more additional proteins, of catalyzing an oxidation step in the biological pathway for conversion of cholesterol into hydrocortisone, which step is selected from the group consisting of: the conversion of cholesterol to pregnenolone;

the conversion of pregnenolone to progesterone;

the conversion of progesterone to 17 α -hydroxyprogesterone;

the conversion of 17 α -hydroxyprogesterone to cortexolone; and the conversion of cortexolone to hydrocortisone

or from the group comprising the natural proteins of the biological pathway for conversion of cholesterol into hydrocortisone being selected from the group consisting of: side-chain cleaving enzyme (P₄₅₀SCC); adrenodoxin (ADX); adrenodoxin reductase (ADR); 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD); steroid-17 α -hydroxylase

(P₄₅₀17 α); NADPH cytochrome P₄₅₀ reductase (RED); steroid-21-hydroxylase (P₄₅₀C21), and steroid-11 β -hydroxylase (P₄₅₀11 β), characterized by heterologous DNA encoding at least two expressible proteins which catalyze at least two separate oxidation steps.

26. A recombinant host cell of Claim 25 wherein the heterologous DNA coding sequences originate from bovine species.
27. A recombinant host cell of Claim 25 wherein the heterologous DNA encodes at least the P₄₅₀17 α and the P₄₅₀C21 proteins.
28. A recombinant host cell and progeny thereof of Claim 25 wherein the host is a micro-organism.
29. A recombinant host cell and progeny thereof of Claim 28 wherein the host is a species selected from the group consisting of Saccharomyces, Kluyveromyces, Bacillus and Escherichia coli.
30. In a process for the preparation of hydrocortisone from sterols, comprising culturing a recombinant cell in a nutrient medium, the improvement comprising a recombinant host containing an expression cassette, operable in a recombinant host, comprising a heterologous DNA coding sequence encoding a protein, which is functional, alone or in cooperation with one or more

additional proteins, of catalyzing at least one oxidation step in the biological pathway for conversion of cholesterol into hydrocortisone, which step is selected from the group consisting of:

the conversion of cholesterol to pregnenolone;
the conversion of pregnenolone to progesterone;
the conversion of pregnenolone to 17 α -hydroxyprogesterone;
the conversion of 17 α -hydroxyprogesterone to cortexolone;
the conversion of cortexolone to hydrocortisone, and the corresponding control sequences effective in said host.

31. An expression cassette of claim 4, wherein the heterologous DNA encodes the enzyme 3 β -HSDH.

32. The process of claim 21, wherein the oxydation is the 3 β -hydroxydeshydrogenation/isomerization of pregnenolone.

33. A recombinant host cell of claim 25, wherein the heterologous DNA encodes at least the P450 17 α and the 3 β -HSDH proteins.